IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application of:	Denise L. Faustman	**ON APPEAL**
Serial No.:	09/913,664	ART UNIT: 1651
Filed:	August 17, 2001	EXAMINER: V. Afremova
Entitled:	METHOD FOR INHIBITING TRANSPLANT REJECTION	

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APPEAL BRIEF PURSUANT TO 37 C.F.R. §41.37

Sir:

Pursuant to 37 C.F.R. §41.37, Appellant submits this Brief on Appeal, setting forth the basis of her appeal from the Office Action mailed September 14, 2005 finally rejecting Claims 1-14 and 16-23 of the above-identified patent application.

Notice of Appeal was filed January 17, 2006.

This Appeal Brief is accompanied by the small entity filing fee under 37 C.F.R. §41.20(b)(1) and a petition for extension of time under 37 C.F.R. §1.136(a) with the appropriate fee under 37 C.F.R. §1.17(a)(5). The Commissioner is hereby authorized to charge any additional fees required in connection with the filing of this Appeal Brief to PTO Deposit Account No. 50-0268.

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REAL PARTY IN INTEREST

Appellant hereby identifies Denise L. Faustman, M.D., Ph.D., as the owner of the invention disclosed in the present application.

RELATED APPEALS AND INTERFERENCES

No related appeals or interference proceedings are known to Appellant or Appellant's legal representatives that are related to the present appeal or that will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

STATUS OF CLAIMS

The present application was filed with Claims 1-37. The present status of all claims is as follows:

Claims 1-14, rejected and on appeal.

Claim 15, canceled.

Claims 16-23, rejected and on appeal.

Claims 24-37, canceled.

The appealed Claims 1-14 and 16-23 appear in the attached Claims Appendix (Tab A).

STATUS OF AMENDMENTS

All of Appellants' amendments have been entered. No amendments have been submitted after final rejection.

SUMMARY OF CLAIMED SUBJECT MATTER

The present invention is directed to a method for inhibiting rejection of transplanted donor tissue by treating viable mammalian allogeneic or xenogeneic transplant tissue with an enzyme so as to **temporarily ablate** MHC Class I antigen complexes from the surface of the transplant tissue, then transplanting the living, treated donor tissue into a host before reappearance of MHC Class I antigen complexes occurs on

said donor tissue. (See, e.g., page 3, line 30, to page 4, line 2, of the specification, and Claim 1.) Ablation of MHC Class I antigen complexes on the donor tissue prevents the immediate immune recognition and attack by cytolytic T cells (CTLs) of the host (such recognition and attack being MHC Class I antigen complex-mediated processes); and thereafter, continued expression by the viable transplant tissue of MHC Class I molecules results in the gradual reappearance of MHC Class I antigen complexes on the surface of the transplant tissue cells. (That is, the ablation of the MHC Class I antigen complexes from the donor tissue is **temporary**. See, page 5, line 32, to page 6, line 2.) The gradual re-expression and re-presentation at the surface of the donor cells of MHC Class I antigen complexes provides the normal mechanism for educating the host's immune system to identify the new (donor) tissue as "self" and to cause deletion of the subpopulation of natural host CTLs capable of recognizing and rejecting the donor tissue. (See, e.g., page 6, lines 6-12, and Example 2, on pages 9-10 of the application.)

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues for consideration in the present appeal are:

I. Whether the methods recited in appealed Claims 1, 2 and 5 are anticipated under 35 U.S.C. §102(b) by Civin, U.S. Pat. No. 5,081,030 ("Civin").

II. Whether the methods recited in appealed Claims 1-9, 12-14, and 16-23 would, at the time of Appellant's invention, have been obvious under 35 U.S.C. §103(a) to a person of ordinary skill in the art of immunology in view of the combined teachings of Civin, taken with Galati et al., *Cytometry*, 27: 77-83 (1997) ("Galati"), Lee et al., U.S. Pat. No. 5,670,358 ("Lee"), and Brownlee et al., U.S. Pat. No. 6,156,306 ("Brownlee").

III. Whether the methods recited in appealed Claims 1-14, and 16-23 would, at the time of Appellant's invention, have been obvious under 35 U.S.C. §103(a) to a person of ordinary skill in the art of immunology in view of the combined teachings of Civin, taken with Galati, Lee, and Brownlee, and further in view of Stone et al., *Transplantation*, 65: 1577-1583 (1998) ("Stone").

ARGUMENTS

The rejection of the appealed claims is in error for the following reasons:

1. The Civin reference cited by the Examiner cannot anticipate Appellant's invention because Civin does not disclose a method for inhibiting rejection of viable donor tissue by a host mammal. Civin does not teach the serial steps of (1) treating viable donor tissue with an enzyme such as papain to temporarily remove (ablate) MHC Class I surface antigens prior to transplantation, (2) transplanting the treated viable tissue into a host mammal, and (3) maintaining the viable donor tissue in the host after transplantation. The Civin reference cannot teach any part of Appellant's invention because that reference relates to the selection of progenitor bone marrow cells, which undifferentiated cells do not yet express MHC Class I molecules. Thus, the reference cannot begin to teach even step (1) of the claimed method, and a rejection for anticipation must necessarily be held in error.

2. The combination of Civin taken with Galati, Lee, and Brownlee fails to suggest the method of Claims 1-9, 12-14, and 16-23 of Appellant's invention because the combined teachings of the references do not suggest a method of inhibiting rejection of transplanted tissue by surface treatment of donor tissue while maintaining viability, transplantation before re-expression of MHC Class I antigens takes place, and maintenance of the viable donor tissue in the host to allow immune system re-education to occur. Instead, the reference combination teaches the harvest of surface molecules as a means of quantitative analysis, cobbled with intracellular blocking of MHC Class I expression. The rejection offers only a hindsight combination of certain elements of Appellant's invention without a logical connection found in the references themselves.

3. Finally, the combination of Civin taken with Galati, Lee, Brownlee, and further in view of Stone fails to suggest the method of Appellant's invention as defined in appealed Claims 1-14 and 16-23. The addition of Stone to the combination of Civin taken with

Galati, Lee, and Brownlee does not render obvious the subject matter of the present application, in particular Claims 10 and 11. Stone teaches pre-transplant treatments rendering the donor tissues non-viable, i.e., such that the treated tissue is rendered incapable of re-expressing any MHC Class I antigens post-transplant. Thus, the addition of the Stone reference takes the reference combination further away from any teaching relevant to Appellant's invention.

The following discussion provides the factual and legal basis for the reasons set forth above for finding error in the final rejection of Appellant's claims.

Introductory: The art cited to reject Appellant's claims is not relevant to the invention as claimed

Generally, the principle of transplantation of live tissue from a donor into a host is known (*see*, paragraph bridging pages 2-3 of Appellant's application), yet the Examiner has resorted to publications that simply disclose the use of known enzymes having known specificities which make them suitable for performing various *in vitro* laboratory methods, then has twisted the teachings of the cited references to argue that in combination they meet the teachings of Appellant's invention, when in actuality the overriding concept of temporary ablation and post-transplant re-expression of MHC Class I antigens is nowhere found in the references, in any combination of their teachings.

Because none of the cited references, individually or combined, can guide a person skilled in this art to a method for successfully inhibiting rejection of living transplant tissues by a host via temporary ablation of MHC Class I molecules on the surface of donor cells followed by reexpression of the donor MHC Class I post-transplantation in the host, the cited references and combinations proposed by the Examiner by definition cannot meet or suggest the invention defined in the appealed claims within the meaning of 35 U.S.C. §102(b) or §103(a). Accordingly, reversal of the rejections based on the cited art is respectfully believed to be in order.

I. The Civin reference does not anticipate the invention of Claims 1, 2, and/or 5 under 35 U.S.C. §102(b)

For anticipation under 35 U.S.C. §102 by a printed publication, that publication must teach each and every element or aspect of the claimed invention. As stated in MPEP §2131:

> **"TO ANTICIPATE A CLAIM, THE REFERENCE MUST TEACH EVERY ELEMENT OF THE CLAIM**
> 'A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference.' *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). 'The identical invention must be shown in as complete detail as is contained in the . . . claim.' *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989)." (emphasis in original)

There are clearly elements set forth in Appellant's claims which are not taught in the Civin publication, hence the rejection for anticipation is in error. The Civin reference discloses a method for harvesting stem cells by means of affinity isolation targeting the developmental stage-specific surface marker MY10. MY10 is present on lymphohematopoetic progenitor cells but not on mature cells:

> "The procedure employs a monoclonal antibody which selectively recognizes human lymphohematopoietic progenitor cells, such as the anti-MY10 monoclonal antibody that recognizes an epitope on the CD34 glycoprotein antigen. Cells expressing the CD34 antigen include essentially all unipotent and multipotent human hematopoietic colony-forming cells (including the pre-colony forming units (pre-CFU) and the colony forming unit-blasts (CFU-Blast)) as well as the very earliest stage of committed B lymphoid cells, but NOT mature B cells, T cells, NK cells, monocytes, granulocytes, platelets, or erythrocytes." (Civin reference at col. 2, lines 10-21)

* * *

> "In a particular embodiment of this invention, cells bearing the CD34 antigen are positively selected to provide a population of cells for bone marrow transplant without any foreign receptor on their surfaces. This cell population contains the lymphohematopoetic progenitor cell types but does not contain mature cells such as mature B cells, T cells, NK cells, monocytes, granulocytes, platelets and erythrocytes, nor does it contain malignant cells. The method of this invention first requires that a population of CD34-positive, receptor-bound cells be obtained based on their ability to bind anti-MY10 antibody. Procedures to prepare such populations bound to the anti-MY10 antibody are taught in U.S. Pat. No. 4,714,680 and are incorporated herein by reference." (Civin reference at col. 5, line 59, to col. 6, line 5.)

Thus, the Civin reference teaches the critical selection of immature cell types for bone marrow transplant. The cells are captured by affinity chromatography utilizing a receptor (e.g., antibody) for a surface ligand (e.g., CD34), then the cells are collected by enzymatic degradation of the surface ligand, so as to leave no foreign antigenic structures attached to the collected cells:

> "After the cell-receptor complex is separated, the cells are released from the complex by treatment with a degradative enzyme, where the enzyme specifically degrades the cell surface ligand to which the receptor is bound without substantially decreasing the viability or function of the cell population." (Civin reference at col. 4, lines 55-60.)
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> "The cell population, after enzyme treatment and washing, is made up of the positively selected cell-type and substantially free of foreign, antigenic material. It is therefore particularly suited for therapeutic use.
>
> "In a particular embodiment of this invention, cells bearing the CD34 antigen are positively selected to provide a population of cells for bone marrow transplant without any foreign receptor on their surfaces. This cell population contains the lymphohematopoetic progenitor cell types but

> does not contain mature cells . . ." (Civin, at col. 5, lines 54-64.)
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> * * *
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> "By attacking the cell surface 'ligand,' rather than the receptor on the affinity matrix, the cells are freed of the 'foreign material' which had coated their surfaces . . .The resulting cell suspension is substantially free of receptor material." (Civin, at col. 3, lines 37-42.)

The progenitor cells that are the object of the Civin procedure are immature cells that do not yet exhibit MHC Class I antigen complexes on their surfaces. See, e.g., S. Itescu, "Stem Cells and Tissue Regeneration: Lessons From Recicpients of Solid Organ Transplantation," Appendix L in Monitoring Stem Cell Research (The President's Council on Bioethics; Jan. 2004), pp. 2, 9., http://bioethicsprint.bioethics.gov/reports/stemcell/appendix_l.html (*see*, Evidence Appendix at Tab B); M. Gabbianelli et al., "HLA Expression in Hemopoietic Development," *J. Immunol.*, vol. 144: 3354-60 (1990) (*see*, Evidence Appendix at Tab C). This fact of the MHC Class I-negative phenotype of progenitor cells is submitted to be common knowledge to the person of ordinary skill in the art of immunology. Accordingly, as a matter of *fact*, the Civin reference cannot be interpreted as teaching any method which has as its first step "treating viable donor tissue with an enzyme effective for temporarily ablating MHC Class I antigens from said donor tissue," as is expressly recited in Claim 1 of the appealed claims. Since appealed Claims 2 and 5 likewise incorporate the recitations of treating transplant tissue to ablate MHC Class I antigens, Civin also fails to meet the features of those claims.

For this reason alone, Civin does not anticipate appealed Claims 1, 2 and 5 of Appellant's application, and the final rejection based on 35 U.S.C. §102(b) over Civin should be reversed.

The Examiner's Arguments in the Final Rejection

The Examiner's statements regarding the teachings of Civin appear to read into the reference more elements than are there, in order to make a reference relating to cell

collection seem to contain a teaching relating to transplantation methods. In the paragraph bridging pages 2-3 of the final Office Action, the Examiner states:

> "US 5,081,030 [Civin] discloses a method for transplantation [of] bone marrow cells wherein the method comprises [the] *step of treating a viable donor tissue with enzyme chymopapain* (col. 11, lines 30-35), [the] step of transplanting the treated viable donor tissue into [a] host mammal (col. 11, line 45) and [the] step [of] maintaining the treated viable donor tissue in the host mammal (col. 11, line 57). The cited patent clearly teaches that cells retain viability after enzymatic treatment. . . . The cited patent teaches that enzymatic treatment is intended to release cell surface molecules and that proteases including chymopapain and papain release cell surface proteins and glycoprotein antigens. *The cited patent is considered to anticipate the claimed invention because it comprises identical active steps* and, thus, the intended effects are reasonably expected to be identical as related to removal of antigens of MHC class I and to inhibition of donor tissue rejection, particularly in view [of the fact] that the cited patent demonstrates better survival of animals [that] received engraftment of enzymatically treated cells." (Office Action of 9/14/05, pp. 2-3; emphasis added.)

First, the Examiner is stretching the Civin reference to fit a novel method for inhibiting transplant rejection, whereas the actual intended teaching of Civin is to provide a means of collecting MHC Class I-negative progenitor cells free of cell surface receptor fragments. That Civin relates to cell collection and not a method of transplantation comes into focus when the claims of the Civin patent are considered. For example, Claim 1 of Civin reads as follows:

> "1. A method for *releasing animal cells bound to antibodies* specific for the MY10 epitope which comprises treating the antibody bound animal cells with chymopapain and separating viable cells from said antibodies." (emphasis added)

From a general consideration of the Civin reference, it is readily seen that the teachings of Civin do not impart a means for preparing donor tissue for transplantation so as to inhibit rejection of the tissue by the host. The progenitor cells that are the target of Civin's method are already suitable for transplant because they lack sufficient differentiation to cause rejection; the object of Civin's teaching is to provide such progenitor cells free of other, more differentiated (and more prone to rejection) cells.

Second, and more important, the Examiner's statement that the Civin patent "comprises identical active steps" in comparison to Appellant's method is clearly wrong. The Examiner characterizes Civin as teaching a method beginning with the step of "treating a viable donor tissue with enzyme chymopapain." (See, quoted passage *supra*.) However, the first step of Appellant's method is "treating viable donor tissue with an enzyme effective for temporarily ablating MHC Class I antigens from said donor tissue." (See, step (a) of Appellant's Claims 1 and 12.) As pointed out above, the Civin reference relates to collection of immature, MHC Class I-negative progenitor cells, and therefore as a reference it is incapable of teaching a step where the critical feature is "temporarily ablating MHC Class I antigens".

The Examiner characterizes the next step of the Civin method as the step of "transplanting the treated viable donor tissue into [a] host mammal," which is different from Appellant's recited next step, i.e., "transplanting said treated, viable donor tissue into said host mammal before MHC Class I antigens are re-expressed on the surface of said donor tissue." (See, step (b) of Appellant's Claims 1 and 12.) It is part of the teaching of the present invention, but not of Civin, that prepared tissues denuded of MHC Class I antigens by enzymatic ablation will later re-express those antigens and become "visible" to the host immune system, and therefore it is an important feature of Appellant's invention to transplant the viable, treated cells before such re-expression of MHC Class I antigens occurs. This feature and this step are absent from the disclosure of Civin.

Finally, the next step of the method of Civin, as characterized by the Examiner, is "maintaining the treated viable donor tissue in the host mammal." According to Civin, the result of this last step is the development of "identifiable hematopoietic cells within a

hypocellular marrow" and "reconstitute[d] hematopoiesis". (See, Civin at col. 11, lines 63-64; col. 12, line 1.) In contrast, the result of the last step in Appellant's method is the survival of the graft tissue as immunologically acceptable tissue. (See, e.g., Appellant's specification at page 6, lines 1-8.) In other words, the Civin reference teaches that cells collected according to the described separation method are capable of maturing and differentiating into various cells of the hematopoietic cell lineage; whereas the present invention teaches that mature cells may be treated to avoid rejection and become tolerated by a host *because* of the re-expression of MHC Class I antigen structures post-transplant.

From the foregoing discussion, it is clear that the Examiner has ascribed to Civin teachings that it does not, and indeed can not, provide to the art. Accordingly, the rejections for anticipation based on Civin are in error and should be reversed by the Board.

II. The combination of Civin taken with Galati, Lee, and Brownlee does not render the subject matter of Claims 1-9, 12-14, and 16-23 obvious under 35 U.S.C. §103(a)

In the final Office Action, the Examiner rejected Claims 1-9, 12-14, and 16-23 under 35 U.S.C. §103(a) as being unpatentable over Civin, taken with Galati, Lee, and Brownlee.

For the reasons set forth above, the teachings of Civin can be seen to be unrelated to the novel methods taught in Appellant's application and, as discussed below, the Galati, Lee, and Brownlee references do not provide a "bridge" linking the teachings of Civin with the present invention, and therefore the citation of the references cannot support a *prima facie* case of obviousness.

The standard for assessing obviousness in view of prior art is well established:

> "The consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood of success viewed in light of the prior art. **Both** the suggestion and the expectation of success **must** be founded in the prior art, **not** in the Applicant's disclosure." *In re Dow Chemical Co.*,

> 837 F.2d 469, 473, 5 U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988)[1] (emphasis added).

The concept of attempting to prevent transplant rejection of viable donor tissue by temporarily ablating the MHC Class I antigen complexes present on the surface of the donor tissue prior to transplantation, while maintaining the ability of the donor tissue to re-express the MHC Class I antigen complexes after transplant, is nowhere found in the cited prior art. As exhaustively reviewed above, the Civin reference only includes teachings related to releasing immature $CD34^+$ cells immobilized on an affinity matrix. No teaching relating to ablation of MHC Class I antigens can be extracted from the Civin reference because $CD34^+$ cells are not sufficiently mature to express MHC Class I antigens.

The secondary Galati reference does not even relate to the field of transplantation. Rather, Galati discloses an *in vitro* flow cytometry method for quantitation of isolated MHC Class I antigen complexes. Galati teaches removing intact MHC Class I antigen complexes from living cells, then collecting the complexes and measuring an acid-isolated component thereof (β_2-microglobulin) as a means of quantitating the expression level of Class I antigen complexes on the original cells. No reference to transplantation of living cells is made in Galati; it is a document relating to laboratory methods.

The secondary Lee reference relates to quenching enzymatic activity in a method wherein viable cells are being liberated from connective tissue by enzymatic digestion. Here again, the Examiner has ascribed to the reference teachings that do not appear anywhere in the reference itself but are transplanted from Appellant's own disclosure. With respect to Lee, the Examiner states:

> "In addition, US 5,670,358 [Lee] is relied upon to demonstrate that hepatocytes and islet cells useful for transplantation are prepared by enzymatic treatment with chymopapain or papain." (Office Action of 9/14/05, p. 5.)

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[1] *citing, Burlington Industries v. Quigg*, 822 F.2d 1581, 1583, 3 U.S.P.Q.2d 1436, 1438 (Fed. Cir. 1987); *In re Hedges*, 783 F.2d 1038, 1041, 228 U.S.P.Q. 685, 687 (Fed. Cir. 1987); *Orthopedic Equipment Co. v. United States*, 702 F.2d 1005, 1013, 217 U.S.P.Q. 193, 200 (Fed. Cir. 1983); *In re Rinehart*, 531 F.2d 1048, 1053-54, 189 U.S.P.Q. 143, 148 (C.C.P.A. 1976).

In fact, the Lee reference actually teaches away from treating cells with papain or chymopapain: The disclosure of the Lee reference provides a means for extinguishing papain or chymopapain activity after such enzymes are used to dissolve connective tissue. More specifically, Lee teaches treating tissue, after digestion with chymopapain or papain to destroy connective tissue, by adding an inhibitor that deactivates the chymopapain or papain, in order to prevent further damage to the viable cells released by the digestion.

That Lee relates to a method for quenching the activity of chymopapain or papain rather than to a method for applying chymopapain or papain for a specific purpose is readily seen by the most cursory review of the Lee reference. See, for example, the title, the abstract, or Claim 1 (reproduced below):

> "1. A process *for inhibiting the activity of an enzyme selected from the group consisting of chymopapain and papain* which comprises adding a polysaccharide selected from the group consisting of glycogen, desulfated heparin, and hyaluronic acid to a medium containing said enzyme, said polysaccharide being added in a concentration effective to inhibit the activity of said enzyme." (emphasis added)

Or, a close review of the document reveals that the thrust of the Lee teaching is to dissolve connective tissue and rescue viable cells from contact with the enzymes, rather than a positive teaching that enzyme contact is a desirable step for treating the cells. For instance, in the Lee patent's Summary of the Invention, it is taught that:

> "An exemplary process of the present invention includes enzymatically digesting connective tissue by providing an enzyme composition containing papain or chymopapain, or a mixture of papain and chymopapain, in an amount sufficient to hydrolyze connective tissue and dissociate desired viable cells from such tissue . . . *It is essential to halt or at least substantially slow down the enzymatic activity* in the medium containing the isolated viable cells *as soon as possible after the cells are dissociated from the tissue* in order to preserve the cell integrity. This is accomplished by preventing excessive digestion . . . Following addition of an inhibitor in accordance with the

> present invention, the viability of the isolated cells is greatly preserved and the yield of viable cells is increased." (See, Lee at col. 2, lines 21-38; emphasis added.)

MHC class I antigens are not mentioned in Lee, nor is there any mention of intentionally cleaving MHC class I antigen complexes from the cell surface for any reason. There is, however, an admonition in Lee to AVOID such cleavage set forth in the beginning of the reference:

> "It is necessary to stop undesired enzymatic activity as quickly as possible once the cells are dissociated from the tissues containing them, especially where isolation and purification procedures may continue for an extended period of time. Otherwise, cell integrity and function may be *compromised by the continuing enzymatic degradation of cell surfaces* which may result in substantial damage to the cells, such as cell membrane degradation or loss of cell surface receptors. (Lee at col. 1, lines 33-41; emphasis added.)

Appellant submits that this amounts to a direct teaching away from the method of the present invention as defined in the claims on appeal, in that cells otherwise identified in Lee as being suitable for transplant are taught as requiring protection from the effects of exposure to chymopapain or papain, whereas in Appellant's claims the direct treatment of transplantable cells with enzymes such as chymopapain or papain in order to temporarily ablate particular cell surface structures is required.

Thus, in contrast to the Examiner's assertion, Lee does not "demonstrate that [cells] useful for transplantation are prepared by enzymatic treatment with chymopapain or papain." In fact, Lee teaches just the opposite, i.e., that cells are prepared for isolation by enzymatic treatment of connective tissue, followed by addition of an inhibitor to prevent enzymatic treatment of the cells.

The Brownlee reference teaches genetic alteration of donor tissue in order to produce permanently MHC class I-negative cells.

> "The invention provides a method and vectors to express a gene, derived from a virus, that blocks the intracellular transport and/or intracellular maturation within the graft of

> proteins called MHC class I products." (Brownlee at col. 5, lines 31-35.)
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> "The present invention provides a method of rendering cells more readily transplantable between histoincompatible individuals . . . The method comprises obtaining a population of cells to be transplanted, e.g., by explantation from a cadaveric donor, and introducing into substantially all of the cells of the population a vector that makes a nucleic acid product." (Brownlee at col. 5, lines 43-49.)

Brownlee represents an approach to transplantation having drawbacks the present invention seeks to overcome. See, e.g., Appellant's specification at page 3, lines 10-25. The Brownlee reference teaches to *genetically alter* cells prior to transplant, in order to permanently block the expression of MHC Class I antigen complexes by the transplant cells. This is in direct contrast to the treatment of the method of the present invention, which requires the *temporary* ablation of MHC Class I antigen complexes from the donor tissue surface, but contemplates the re-expression of such MHC Class I antigen complexes post-transplant. Appellant's invention counts on the return of MHC Class I antigen expression (i.e., for re-education of the host immune system and induction of tolerance to the donor tissue) as much as it does on the initial ablation of MHC Class I antigen complexes for avoidance of acute donor tissue rejection. Thus, it would be impossible to carry out Appellant's invention with materials genetically altered to be MHC Class I-negative in accordance with the teaching of Brownlee.

The Combination of the References

When the teachings of the Civin reference are combined with those of Galati, Lee and Brownlee, it is simply not possible to derive the method of Appellant's invention, even though some isolated facts on which Appellant's invention depends are acknowledged or restated in some of the citations. For example, the known fact that papain cleaves MHC Class I molecules from the surface of cells is repeated in Galati, but

the combined teachings of all the references does not contain the suggestion of cleaving the MHC Class I molecules and retaining the cells for use in transplant.

Likewise, Brownlee recognizes that MHC Class I antigens are a key structure for host immune system recognition, and suppressing expression of MHC Class I in donor cells deprives the host immune system of a recognition target for T cell attack on the donor tissue used in a transplant. However, Brownlee and all the other references fail to recognize the role of the emergence of MHC Class I antigens in the education of the host immune system to tolerate the presence and propagation of the donor cell population. Appellant's claims require that the ability to express MHC Class I antigens is maintained in the enzyme-treated donor cells; by contrast, the reference combination including Brownlee teaches the permanent genetic alteration of the donor cells so as to lose their ability to produce MHC Class I antigens.

The failure of the reference combination to meet or suggest Appellant's invention is clearly seen in the combined teachings relating to the use of enzymes such as chymopapain or papain. In the teaching of the cited references, enzymes are destructive reagents, used to separate desired materials from undesired materials. Thus, in Civin, enzymes capable of cleaving CD34 are used to release cells immobilized by an unwanted (immunogenic) receptor; in Galati, enzymes are used to separate MHC molecules to be quantitatively measured from the unwanted cells on which the MHC molecules were originally expressed; and in Lee, enzymes are used to hydrolyze undesired connective tissue to liberate viable cells, after which the enzymatic activity is quickly quenched before any surface alteration of the liberated cells can take place. *In no sense* does the combination of references teach the use of enzymes as a treatment for living tissue as a necessary step prior to transplantation. Furthermore, it is a distinction of Appellant's invention that enzymes are NOT used for the permanent separation of the cells from an unwanted component; rather, according to Appellant's claims, enzymatic activity is the treatment specified *because* it will not alter the ability of the MHC molecule enzymatically ablated to re-emerge as a function of the continued viability of the otherwise unaltered cells.

In view of the foregoing remarks, it is clear that the essential teaching of the present invention of a treatment for donor tissues so as to inhibit donor tissue rejection is not met and is not suggested by the combination of Civin, taken with Galati, Lee, and Brownlee. Therefore, the final rejection under 35 U.S.C. §103(a) based on the combination of Civin, taken with Galati, Lee, and Brownlee should be reversed by this Board.

Elements of appealed Claims 12-14 and 16-20 additionally not suggested by the combination of Civin taken with Galati, Lee, and Brownlee

In addition to the reasons given above, the subject matter of appealed Claims 12-14 and 16-20 cannot be suggested by the combination of Civin taken with Galati, Lee, and Brownlee. Claims 12-14 and 16-20 include the particular step of Claim 12, i.e., "(d) transplanting a second donor tissue into said host animal." This second transplantation relates to the pre-tolerization embodiments of the present invention discussed, e.g., at page 6, lines 6-12, of the specification. A secondary transplantation is not suggested by Civin and is certainly not suggested by Lee as implied by the Examiner. Civin and Lee relate to methods of collecting cells and not to transplantation techniques. Furthermore, since Galati does not relate at all to the field of transplantation and makes no mention of transplantation, Galati likewise cannot supplement Civin and Lee; likewise the Brownlee reference, teaching genetic alteration of cells, is irrelevant to the invention of Claim 12 and its dependent claims.

The combination of Civin, taken with Galati, Lee, and Brownlee, does not suggest the two-stage transplantation method recited in Claims 12-14 and 16-20. Accordingly, in addition to the failures of the reference combination applicable to all the appealed claims, the reference combination also fails to render obvious the particular embodiments of Claims 12-14 and 16-20.

For the foregoing additional reason, Claims 12-14 and 16-20 are not obvious under 35 U.S.C. §103(a), and their final rejection should be reversed by this Board.

III. The combination of Civin taken with Galati, Lee, and Brownlee, and further in view of Stone, does not render the subject matter of Claims 1-14 and 16-23 obvious under 35 U.S.C. §103(a)

The other final rejection based on obviousness under 35 U.S.C. §103(a) is identical to the rejection discussed in section II, above, except that the Stone reference has been added mechanically to tie the elaborate reference combination to specific subject matter (i.e., α-galactosidase) recited in appealed Claims 10 and 11. In the final Office Action, the Examiner states that the rejection of Claims 1-9, 12-14, and 16-23 is according to the previously asserted combination (see section II, above) but that Claims 10 and 11 recite additional subject matter not suggested by the previous combination.

> "Claims 1-9, 12-14, and 16-23 as explained above. Claims 10 and 11 are further drawn to the use of combination of papain and alpha-galactosidase in the method for inhibiting transplant rejection." (See, Office Action of 9/14/05, p. 6.)
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>
> "The reference by Stone et al. discloses a method of inhibiting transplant [rejection] wherein the method comprises [the] step of treating donor tissue with galactosidase and [the] step of transplanting the treated tissue in to a host recipient and wherein the method results in a reduction of inflammatory reaction or immune response of [the] host." (See, Office Action of 9/14/05, p. 7.)
>
> * * *
>
> "The reference Stone et al. was/is relied upon to demonstrate that glycosidase such as or alpha-galactosidase is known to remove alpha-gal epitopes from xenografts in order to alter or to reduce immune response of [a] host recipient upon transplantation." (See, Office Action of 9/14/05, p. 11.)

The Stone reference does indeed disclose a method of treating cartilaginous tissue intended for transplant with α-galactosidase prior to transplanting the cartilage, however

the method described in Stone is for the transplant of non-viable cartilage plugs which are not subject to the hyperacute rejection which characterizes the regular transplantation of (non-syngeneic) living tissue. As stated directly in the Stone reference:

> "Because porcine *cartilage* does not undergo hyperacute rejection [endnote omitted], studies of the immune response to such xenografts also can provide important information on the characteristics of chronic xenograft rejection in primates and humans. This information has not been available from organ xenografts due to the hyperacute rejection response to *organ* transplantation [endnote omitted]." (Stone at page 1578, left column.)

It is seen that the Stone reference itself makes a distinction between transplantation of living organs (i.e., viable tissue, as in Appellant's invention) and the comparatively inert cartilaginous tissue dealt with in the Stone reference (i.e., alcohol-treated, non-viable cartilage plugs).

As Appellant has pointed out exhaustively in arguing against previous rejections in this application where the primary references were two patents to Oliver (U.S. 4,399,123 and U.S. 5,397,353) involving transplanting killed pig dermis, combined with the same Galati reference and the same Stone reference, there is no teaching relevant to Appellant's invention provided by methodologies that first treat the donor tissue so as to render it non-viable. As pointed out in the previously filed Declaration of Denise L. Faustman Under 37 C.F.R. §1.132, filed March 24, 2004, the preparation of cartilage according to the cited Stone document results in 100% non-viable tissue.

It is acknowledged that the Stone reference is relied on solely "to demonstrate that glycosidase . . . is known to remove alpha-gal epitopes from xenografts in order to alter or to reduce immune response of [a] host recipient upon transplantation," however it is pointed out that the particular xenograft of Stone is non-viable and is not subject to hyperacute rejection by the host. Therefore, the Stone teaching is incompetent for the purpose the Examiner cites it. In other words, Stone has no application to "a method of *inhibiting rejection* . . . of donor tissue . . . which is transplanted into [a] host mammal," as is claimed in the present invention, since the killed cartilage plugs of Stone *are not*

donor tissue subject to rejection. Likewise, since Stone is not concerned with and makes no mention of ablation of MHC Class I antigens from donor tissue, it does not cure the failure of the Civin, Galati, Lee and Brownlee references to suggest even the critical first step of Appellant's methods.

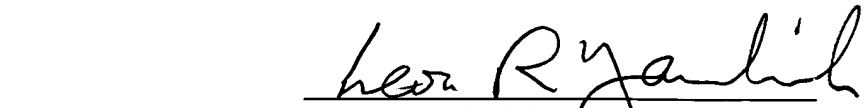
For the foregoing reasons, the Stone reference cannot overcome the deficiencies already noted above for the combination of Civin, taken with Galati, Lee and Brownlee. Since the α-galactosidase treatment of Stone is applied to non-viable tissue, use of the Stone reference in the combination adds nothing to suggest Appellant's method for treating viable tissue or to bring the rest of the combined references closer to suggesting the subject matter of Appellant's claims. Therefore, the final rejection under 35 U.S.C. §103(a) based on the combination of Civin, taken with Galati, Lee, and Brownlee, further in view of Stone, should be reversed by this Board.

CONCLUSION

In asserting that Appellant's claims are anticipated or obvious, the Examiner has found references in the prior art that show the application of an enzyme to cells or tissues that are intended for transplant, but the effects of the enzyme and *the coordination of the impermanent enzyme effects with the act of transplantation* –which is called for by Appellant's claims– finds no expression in any of the references or combination thereof. The central principle of Appellant's invention, that is, the temporary removal of MHC Class I antigens to avoid acute rejection *followed by* the return of MHC Class I antigens after transplantation, to participate in the immunological re-education of the host, is COMPLETELY missing from any combination of the references and is left, instead, to the imagination of the hypothetical person of ordinary skill in the art. This is the quintessential example of the difference between the subject matter sought to be patented and the teachings of the prior art being *too great* for a person having ordinary skill in the art of immunology to navigate, without reference to Appellant's specification.

There is nothing in the references cited by the Examiner in the final rejection that meets all the steps of the invention or prompts a person of ordinary skill in the art to discover the method defined in the claims on appeal. Accordingly, all rejections applied against the claims on appeal are believed to be in error, and reversal of the rejections is respectfully solicited.

Respectfully submitted,



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CERTIFICATE OF MAILING

The undersigned hereby certifies that this paper is being deposited with the U.S. Postal Service as First Class mail under 37 C.F.R. §1.8, postage prepaid, in an envelope addressed to: Mail Stop Appeal Brief - Patent, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date indicated below:

August 16, 2006 — date

Nasim G. Memon

Claims on Appeal

1. A method for inhibiting rejection by a host mammal of donor tissue from another mammal which is transplanted into the host mammal, said method comprising
(a) treating viable donor tissue with an enzyme effective for temporarily ablating MHC Class I antigens from said donor tissue,
(b) transplanting said treated, viable donor tissue into said host mammal before MHC Class I antigens are re-expressed on the surface of said donor tissue, and
(c) maintaining said viable donor tissue in said host.

2. The method according to Claim 1, wherein said donor tissue is from a mammal that is the same species as said host mammal.

3. The method according to Claim 1, wherein said donor tissue is from a mammal that is of a different species than said host mammal.

4. The method according to Claim 1, wherein said host mammal is a human.

5. The method according to Claim 1, wherein said tissue comprises blood cells, neurons, hepatocytes, cardiac cells, genetically modified cells, skin cells, precursor cells, endothelial cells, fibroblasts, myoblasts, islets of Langerhans cells, or bone marrow cells.

6. The method according to Claim 1, wherein said tissue is an organ or part of an organ.

7. The method according to Claim 6, wherein said organ is selected from the group consisting of skin, kidney, heart, pancreas, brain, and liver.

8. The method according to Claim 1, wherein said donor tissue is additionally treated with a second enzyme effective to remove an antigenic surface structure from said donor tissue.

9. The method according to Claim 1, wherein said enzyme is papain.

10. The method according to Claim 8, wherein said second enzyme is α-galactosidase.

11. The method according to Claim 8, wherein said donor tissue is treated with a combination of papain and α-galactosidase.

12. A method for inhibiting rejection by a host mammal of donor tissue from another mammal which is transplanted into the host mammal, said method comprising:
(a) treating a first viable donor tissue with an enzyme effective for temporarily ablating MHC Class I antigens from said donor tissue,
(b) transplanting said treated, viable donor tissue into said host mammal before MHC Class I antigens are re-expressed on the surface of said donor tissue, and
(c) maintaining said viable donor tissue in said host mammal, and
(d) transplanting a second donor tissue into said host mammal.

13. The method according to Claim 12, wherein said first donor tissue is donor lymphocytes.

14. The method according to Claim 12, wherein said second donor tissue is also treated prior to transplantation with an enzyme effective for removing MHC Class I antigens from said tissue.

16. The method according to Claim 12, wherein said first and second donor tissue is from a mammal that is the same species as said host mammal.

17. The method according to Claim 12, wherein said first and second donor tissue is from a mammal that is of a different species than said host mammal.

18. The method according to Claim 12, wherein said host mammal is a human.

19. The method according to Claim 12, wherein said first and second donor tissue independently comprises blood cells, neurons, hepatocytes, cardiac cells, genetically modified cells, skin cells, precursor cells, endothelial cells, fibroblasts, myoblasts, islets of Langerhans cells, or bone marrow cells.

20. The method according to Claim 12, wherein said first and second donor tissue is an organ or part of an organ.

21. The method according to Claim 20, wherein said organ is selected from the group consisting of skin, kidney, heart, pancreas, brain, and liver.

22. The method according to Claim 1, wherein said donor tissue is treated with a solution of papain at 5-60 mg/ml for a period of 5 minutes to 24 hours.

23. The method according to Claim 22, wherein said solution contains 20-28 mg/ml papain and said tissue is treated for 30-120 minutes.

EVIDENCE APPENDIX

Monitoring Stem Cell Research

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The President's Council on Bioethics
Washington, D.C.
January 2004
www.bioethics.gov

Appendix L

Stem Cells and Tissue Regeneration: Lessons From Recipients of Solid Organ Transplantation

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CONTENTS

1. OVERVIEW

The Major Histocompatibility Complex (MHC) is located on the short arm of chromosome 6 in humans and encodes the alloantigens known as Human Leukocyte Antigens (HLA), polymorphic cell surface molecules which enable the immune system to recognize both self and foreign antigens. The class II HLA molecules (HLA-DR, HLA-DP, and HLA-DQ) are usually found only on antigen-presenting cells such as B lymphocytes, macrophages, and dendritic cells of lymphoid organs, and initiate the immune response to foreign proteins, including viruses, bacteria, and foreign HLA antigens on transplanted organs.

Following binding of foreign proteins, class II HLA on antigen-presenting cells activate CD4+ T cells, which in turn activate cytotoxic CD8+ T cells to recognize the same foreign antigen bound to HLA class I (HLA-A, HLA-B, and HLA-C, molecules found on the surface of all cells) and destroy the target. The actual recognition of foreign HLA transplantation antigens by T cells is referred to as allorecognition. Two distinct pathways of allorecognition have been described, direct and indirect. The direct pathway involves receptors on the host T cells that directly recognize intact HLA antigens on the cells of the transplanted organ. The indirect pathway requires an antigen-presenting cell that internalizes the foreign antigen and presents it via its own HLA class II molecule on the surface of an antigen-presenting cell to the CD4+ helper T cells.

Once recognition has taken place, an important cascade of events is initiated at the cellular level, culminating in intracellular release of ionized calcium from intracellular stores. The calcium binds with a regulatory protein called calmodulin, forming a complex that activates various phosphatases, particularly calcineurin. Calcineurin dephosphorylates an important cytoplasmic protein called nuclear factor of activated T cells (NFAT), resulting in its migration to the nucleus and induction of the production of various cytokines such as IL-2. These cytokines recruit other T cells to destroy the transplanted organ, ultimately resulting in rejection and loss of the graft.

Immunosuppressive regimens used to prevent allograft rejection are aimed at inhibiting the various arms of the immune response, typically require multiagent combinations, and need to be maintained for the duration of life. The currently used armamentarium confers significant side-effect risks, including infectious and neoplastic complications. Moreover, despite success at preventing early allograft rejection, long-term survival of transplanted organs remains difficult to achieve and novel methods to achieve long-term tolerance are being actively sought.

Stem cells obtained from embryonic or adult sources differ from other somatic cells in that they express very low levels of HLA molecules on their cell surfaces. This endows these cell types with the theoretical potential to escape the standard mechanisms of immune rejection discussed above. However, under conditions that enable cellular differentiation in vitro and in vivo each of these stem cell populations acquires high level expression of HLA molecules, suggesting that their long-term survival following transplantation in vivo may be limited by typical immune rejection phenomena. Recent experimental data, however, provide striking counterintuitive examples that stem cells from both embryonic and adult sources may evade the recipient's immune system and result in long-term engraftment in the absence of immunosuppression despite acquisition of surface HLA molecule expression. These observations may have significant impact on the emerging field of regenerative medicine.

2. IMMUNOBIOLOGY OF ORGAN TRANSPLANTATION

The Human Leukocyte Antigens (HLA)

Differences between individuals which enable immune recognition of non-self from self are principally due to the extreme polymorphism of genes in the Major Histocompatibility Complex (MHC) on chromosome 6 in man which encode the cell surface HLA molecules. These molecules are cell surface glycoproteins whose biologic function is to bind antigenic peptides (epitopes) derived from viruses, bacteria, or cancer cells, and present them to T cells for subsequent immune recognition. Each HLA gene includes a large number of alleles and the peptide binding specificity varies for each different HLA allele. The 1996 WHO HLA Nomenclature Committee report lists more than 500 different HLA class I and class II alleles.

Crystallographic x-ray studies have demonstrated that the hypervariable regions encoded by polymorphic regions in the alleles correspond to HLA binding pockets which engage specific "anchor" residues of peptide ligands. One HLA molecule will recognize a range of possible peptides, whereas another HLA molecule will recognize a different range of peptides. Consequently, no two individuals will have the same capability of stimulating an immune response, since they do not bind the same range of immunogenic peptides. It is estimated that >99% of all possible peptides derived from foreign antigens are ignored by any given HLA molecule. Since in the absence of HLA polymorphism a large number of immunogenic peptides would not be recognized, the extensive HLA polymorphism in the population reduces the chance that a given virus or bacterium would not be recognized by a sizable proportion of the population, reducing the likelihood of major epidemics or pandemics.

T Cell Recognition Of Antigen Presented By HLA Molecules

Since HLA molecules regulate peptide display to and activation of the immune system, considerable effort has been devoted to understanding the molecular basis of peptide-HLA interactions. These issues are important for defining the biology of T cell antigen recognition and the properties of a protein that make it immunogenic or non-immunogenic. Specific antigen recognition by T cells is dependent on recognition by the T cell receptor of a three-dimensional complex on the surface of antigen-presenting cells (APC) comprised of the HLA molecule and its bound peptide. The peptides are produced by complex antigen processing machineries within the APC (i.e. proteolytic enzymes, peptide transporters and molecular chaperones) which generate a pre-selected peptide pool for association with the HLA molecules. The different types of T cells require different HLA molecules for antigen presentation, so-called "HLA restriction" phenomena. T cell receptors on CD8+ cytotoxic T cells (CTLs) bind peptides presented by HLA class I molecules, whereas CD4+ T helper cells (Th) recognize peptides bound to HLA class II molecules. Of the 8-13 amino acid residues of a bound peptide within a class I or II HLA molecule, only three to four amino acid side chains are accessible to the T cell receptor, and a similar number of amino acids are involved in binding to the HLA molecule.

Thymic Education Of T Cells

T cells mature in the thymus to appropriately respond to foreign pathogens without inadvertently attacking the host. Under the influence of various thymic resident cells and factors they elaborate, maturing T cells fall into two categories: those that are able to discriminate between self and non-self and can appropriately respond to foreign pathogens without inadvertently attacking the host, and those which are unable to appropriately discriminate between self and non-self. Dendritic cells have been implicated in the deletion, or inhibition, of T cells reactive to self-antigens, particularly in the thymus during T cell development or in peripheral lymphoid organs. The process of self/non-self discrimination by the maturing T cells is dependent on thymic dendritic cell (DC) presentation of self-antigens in the context of self-HLA molecules. When maturing thymic T cells are highly reactive with self-antigen/HLA complexes, they are deleted so that potentially autoreactive T cells will not be released into the periphery. If a particular foreign antigen can be presented in such a way in the thymus as to fool the maturing T cells into believing that the antigen is part of self tissue, then T cells capable of reacting with

this antigen will also be eliminated. Indeed, it has been demonstrated that when mouse thymic DC present transgenically introduced foreign antigens to developing T cells, the mature peripheral T cell repertoire of the mouse lacks T cells capable of reacting with the specific foreign antigen, i.e. it is tolerant to the foreign antigen. This has raised the possibility that injection of dendritic cells into an allogeneic recipient might induce tolerance to a subsequent allograft by causing deletion or inhibition of alloreactive T cells.

T Cell Recognition Of Alloantigens

Recognition of foreign, or allogeneic, HLA antigens by the recipient immune system is the major limitation to the survival of solid organ grafts. The central role of HLA molecules in allograft rejection is due to their role as restriction elements for T cell recognition of donor antigens and the extensive polymorphism displayed by the HLA molecules, which elicit host immune responses. Although progress has been made in the short-term survival of transplants, chronic immunologic rejection remains an impediment to long-term survival.

The primary cause of acute rejection of transplanted organs is so-called "direct" recognition of whole allogeneic HLA antigens by receptors on the surface of recipient T cells. The direct recognition pathway involves recognition by recipient T cells of donor HLA class I and class II molecules, resulting in the generation of cytotoxic and helper T lymphocytes which play a pivotal role in the rejection process. In contrast, chronic rejection of transplanted organs results from so-called "indirect recognition" of donor HLA peptides derived from the allogeneic HLA molecules shed by the donor tissue. These foreign HLA molecules are taken up and processed by recipient antigen presenting cells (APC), and peptide fragments of the allogeneic HLA molecules containing polymorphic amino acid residues are bound and presented by recipient's (self) HLA molecules to recipient (self) T cells. Although direct and indirect recognition of alloantigen generally leads to adverse graft outcome, tolerance induction may occur following exposure of the recipient to donor alloantigens prior to transplantation. Since this strategy is based on the nature and dose of the antigen as well as the route of administration, understanding how to control the balance between activation and unresponsiveness mediated by the direct and/or indirect recognition of alloantigen is a an area of active research which could lead to development of new therapies to prolong graft survival.

Indirect allorecognition has been implicated in recurrent rejection episodes in various transplantation models of cardiac, kidney and skin grafts. Determinants on donor HLA molecules can be divided into two main categories: (a) the dominant allodeterminants that are efficiently processed and presented to alloreactive T cells during allograft rejection; and (b) the cryptic allodeterminants that are potentially immunogenic but do not normally induce alloreactive responses, presumably due to incomplete processing and/or presentation. Indirect recognition of allo-HLA peptides is important for the initiation and spreading of the immune response to other epitopes within the allograft. So-called "spreading" of indirect T cell responses to other allo-HLA epitopes expressed by graft tissue is strongly predictive of recurring episodes of rejection. Tolerance induction to the dominant donor determinants represents potential effective strategy for blocking indirect alloresponses and ensuring long-term graft survival in animal models.

Tolerance Induction

Advances in surgical methods and current immunosuppressive therapies have led to significant improvement in short-term graft survival, however long-term survival rates remain poor. For example, whereas both kidney and heart allografts have one-year graft survival rates of 85 to 95 percent, only about 50% of transplanted hearts survive five years and only about 50% of kidney grafts survive ten years. Thus, despite being able to achieve short-term success, these relatively poor long-term graft

survival rates demonstrate the limitations of the current clinical immunosuppressive regimens to enable long-term immune evasion by the graft. Consequently, a major goal of transplantation immunobiologists is to induce donor-specific tolerance, allowing the long-term survival of human allografts without the need of HLA-compatibility and without the continuous recipient immunosupression leading to the concomitant risks of infection, malignancy, and/or other specific drug side effects. This would theoretically improve long-term graft survival, reduce or eliminate the continuing need for expensive, toxic and non-specific immunosuppressive therapy and enhance the quality of life.

Insight into some of the mechanisms involved in tolerance induction has been gained from pre-clinical and clinical studies in numerous animal models and in patients, particularly those with liver allografts which typically do not induce a prominent immune response leading to rejection. One possible mechanism by which liver transplantation results in allograft tolerance tolerance may be that the donor or "passenger" lymphoid cells in the transplanted liver emigrate and take up residence in the recipient's immune organs, such as the thymus or lymph nodes. Donor lymphocytes at these sites might "re-educate" the recipient immune system so that the donor organ is not recognized as foreign. In an attempt to initiate a similar process in other organ recipients, transfusions of donor blood or bone marrow have been used to enhance solid organ graft survival in animal models and in clinical trials. These studies are currently ongoing in various organ systems.

Molecular understanding of the cellular immune response has led to new strategies to induce a state of permanent tolerance after transplantation. Several approaches have shown promise, including the use of tolerizing doses of class I HLA-molecules in various forms for the induction of specific unresponsiveness to alloantigens, and the use of synthetic peptides corresponding to HLA class II sequences. Other approaches include alteration in the balance of cytokines that direct the immune response away from the TH1 type of inflammatory response and graft rejection to the TH2 type of response that might lead to improved graft survival, and the use of agents to induce "co-stimulatory blockade" of T cell activation. This latter approach is based on the concept that blockade of a "second signal" to the T cell enables the signal provided to the T cell receptor by the HLA-peptide complex to induce antigen specific tolerance.

The experimental use of human dendritic cells as tolerogenic agents has been limited due to the low frequency of circulating dendritic cells in peripheral human blood, the limited accessibility to human lymphoid organs, and the terminal state of differentiation of circulating human dendritic cells making their further expansion ex vivo difficult. Dendritic cells are migratory cells of sparse, but widespread, distribution in both lymphoid and non-lymphoid tissues. Although the earliest precursors are ultimately of bone marrow origin, the precise lineage of dendritic cells is controversial and includes both myeloid-derived and lymphoid-derived populations. Recent work has revealed that an expanded population of mature human dendritic cells can be derived from non-proliferating precursors in vitro is by culturing bone-marrow derived cells with a combination of cytokines. This method of enrichment for human dendritic cells from a precursor population can result in the production of dendritic cells that are tolerogenic to foreign antigens. Whether such cells could be useful when co-administered with an allograft transplant remains to be determined. Nevertheless, it is clear that considerable progress has been made in the past few years using approaches to manipulate the immune response to enable routine donor-specific tolerance, and there is reason to be optimistic that with better understanding of molecular and cellular mechanisms this goal could be attained.

3. IMMUNOSUPPRESSIVE AGENTS COMMONLY USED IN ORGAN TRANSPLANT RECIPIENTS: BENEFITS AND ADVERSE OUTCOMES

Cyclosporine

Cyclosporine has been the single most important factor associated with improved outcomes after organ transplantation over the past two decades. CyA binds to a cytosolic cell protein, cyclophilin (CyP). The CyA-CyP complex then binds to calcineurin and subsequently blocks interleukin-2 (IL-2) transcription. The binding of IL-2 to the IL-2 receptors on the surface of T lymphocytes is a key stimulant in promoting lymphocyte proliferation, activation, and ultimately allograft rejection. A review of the first decade of experience with heart transplantation revealed a total of 379 cardiac allograft recipients worldwide; actuarial survival rates in this cohort of patients at 1 year and 5 years were 56% and 31% respectively; the main causes of death being acute rejection and the side effects of immunosuppression. With the introduction and widespread use of CyA over the next decade, survival rates dramatically improved to 85% and 75% at 1 and 5 years respectively. Similar results were obtained with other organ transplants, including kidney and lung.

The major adverse effects of CyA are nephrotoxicity, hypertension, neurotoxocity and hyperlipidemia; less common side effects include hirsuitism, gingival hyperplasia and liver dysfunction. CyA nephrotoxicity can manifest as either acute or chronic renal dysfunction. It is important to note that a number of drugs commonly used in transplant patients, such as aminoglycosides, amphotericin B and ketoconazole can potentiate the nephrotoxicity induced by CyA. More than half the patients receiving CyA will require treatment for hypertension within the first year following transplantation. Corticosteroids also potentiate the side effects of CyA such as hypertension, hyperlipidemia and hirsuitism. Frequent monitoring of the serum level is essential to minimize the adverse effects. One of the major limitations of the original oil-based CyA formulation (Sandimmune) is its variable and unpredictable bioavailability. In the mid-90s Neoral was introduced, a new microemulsion formula of CyA, which has greater bioavailability and more predictable pharmacokinetics than Sandimmune.

Tacrolimus

Tacrolimus (FK506) is a macrolide antibiotic that inhibits T-cell activation and proliferation and inhibits production of other cytokines. The product of *Streptomyces tsurubaensis* fermentation, FK 506 was first discovered in 1984 and first used in clinical studies in 1988 at the University of Pittsburgh. While the mechanism of action of tacrolimus is similar to that of CyA, and comparative clinical trials have suggested similar efficacy, it has been suggested that some groups of patients may benefit from tacrolimus rather than CyA as primary immunosuppressive therapy. Unlike CyA, hirsuitism and gingival hyperplasia occur infrequently with tacrolimus; thus, tacrolimus-based therapy may improve compliance and quality of life in female and pediatric transplant recipients. It should be noted that alopecia has been documented with tacrolimus, but is known to improve with dose reductions. The decreased incidence of hypertension and hyperlipidemia with tacrolimus makes it preferable to CyA in patients with difficult to treat hypertension or hyperlipidemia. A final indication for tacrolimus has been as a rescue immunosuppressant in cardiac transplant recipients on CyA with refractory rejection or intolerance to immunosuppression (severe side effects). Since tacrolimus is metabolized using the same cytochrome P450 enzyme system as CyA, drug interactions are essentially the same. Thus, drugs that induce this system may increase the metabolism of tacrolimus, thereby decreasing its blood levels. Conversely, drugs that inhibit the P450 system decrease the metabolism of tacrolimus, thereby increasing its blood levels. It is important to note that some studies have indicated a higher incidence of nephrotoxicity with tacrolimus as compared to CyA.

Azathioprine and Mycophenolate Mofetil (MMF)

Despite being available for more than 35 years, azathioprine is still a useful agent as an immunosuppressive agent. Following administration, azathioprine is converted into 6-mercaptopurine, with subsequent transformation to a series of intracellularly active metabolites. These inhibit both an early step in *de novo* purine synthesis and several steps in the purine salvage pathway. The net effect is

depletion of cellular purine stores, thus inhibiting DNA and RNA synthesis, the impact of which is most marked on actively dividing lymphocytes responding to antigenic stimulation. In currently used immunosuppressive protocols, azathioprine is used as part of a triple therapy regimen along with CyA or tacrolimus and prednisone. Mycophenolate mofetil (MMF), which is rapidly hydrolyzed after ingestion to mycophenolic acid, is a selective, noncompetitive, reversible inhibitor of onosine monophosphate dehydrogenase, a key enzyme in the de novo synthesis of guanine nucleotides. Unlike other marrow-derived cells and parenchymal cells that use the hypoxanthine-guanine phosphoribosyl transferase (salvage) pathway, activated lymphocytes rely predominantly on the de novo pathway for purine synthesis. This functional selectivity allows lymphocyte proliferation to be specifically targeted with less anticipated effect on erythropoiesis and neutrophil production than is seen with azathioprine.

Early studies in human kidney and heart transplant recipients showed that MMF, when substituted for azathioprine in standard triple-therapy regimens, is well tolerated and more efficacious than azathioprine. In a large, double-blind, randomized multicenter study comparing MMF versus azathioprine (with CyA and prednisone) involving 650 patients, the MMF group was associated with significant reduction in mortality as well as a reduction in the requirement for rejection treatment. However, there was noted to be an increase in the incidence of opportunistic viral infections in the MMF group. The overall greater efficacy of MMF compared to azathioprine has resulted in MMF generally replacing azathioprine in triple immunosuppressive protocols together with steroids and cyclosporine in most solid organ recipients.

Corticosteroids

Steroids are routinely used in almost all immunosuppressive protocols after organ transplantation. The metabolic side effects of steroids are well known and lead to significant morbidity and mortality in the post-transplant period. Almost 90% of organ recipients continue to receive prednisone at 1-year post-transplant and 70% at three-years post-transplant. A recent review of over 1800 patients from a combined registry outlined the morbid complications that patients suffer within the first year after transplantation. Many of these complications are known side effects of prednisone, including hypertension (16%), diabetes mellitus (16%), hyperlipidemia (26%), bone disease (5%) and cataracts (2%). It is thereby obvious that avoidance of steroids may decrease morbidity and mortality after organ transplantation. Two general approaches are used to institute prednisone-free immunosuppression: early and late withdrawal.

Withdrawal of prednisone during the first month post-transplant has resulted in long-term success of steroid withdrawal in 50–80% of patients. In these studies, the use of antilymphocyte antibody induction therapy appears to increase the likelihood of steroid withdrawal. Several centers have reported their results with immunosuppressive regimens that did not include steroids in the early post-transplant period. Studies reporting high success rates of 80% have used specific enrolment criteria, such as excluding patients with recurrent acute rejections or those with female gender. Review of numerous studies demonstrate that steroid free maintenance immunosuppression is possible in atleast 50% of patients, is as safe as triple drug therapy and may reduce some of the long-term complications of steroids. Owing to the fact that the majority of acute rejection episodes occur in the first three months post-transplant, steroid withdrawal is made after this time period, resulting in long-term success in about 80% of patients. Generally, there is no need for conventional induction agents when late withdrawal of steroids is done.

Anti-Lymphocyte Antibody Therapy

Despite the extensive use of induction therapy using anti-lymphocyte antibody in solid organ transplantation, their exact role is unclear. There is no doubt that routine use of these agents is

unwarranted as the generalized immunosuppression induced by then increased the risk of infections and malignancy. Despite the lack of consistent data supporting the routine use of induction therapy with anti-lymphocyte antibody agents, there is a role in certain select situations. Specifically, patients with early post-operative renal or hepatic dysfunction may benefit especially by the avoidance of cyclosporine therapy while using these induction agents. Anti-lymphocyte antibody therapy can provide effective immunosuppression for atleast 10 to 14 days without CyA or tacrolimus therapy. It has also been suggested that patients with overwhelming postoperative bacterial infections or diabetics with severe postoperative hyperglycemia may benefit from the comparatively low doses of corticosteroids required during anti-lymphocyte induction therapy.

The two main types of induction agents have been either the polyclonal antilymphocyte or antithymocyte globulins and more recently the murine monoclonal antibody OKT3. While these agents have been shown to be effective in terminating acute allograft rejection and in treating refractory rejection, the results of comparative studies of outcomes with and without monoclonal induction therapy have varied, with most studies demonstrating an effect on rejection that is maintained only while antibody therapy is ongoing. Without repeated administration, these agents only delay the time to a first rejection episode without decreasing the overall frequency or severity of rejection. More importantly, their use has been associated with an increased risk of short-term (infections) and long-term (lympho-proliferative disorders) complications. A complication specific to OKT3 is the development of a "flu-like syndrome" characterized by fever, chills and mild hypotension, typically seen with the first dose.

Since antilymphocyte antibodies are produced in nonhuman species, their use is associated with the phenomenon of sensitization, leading to decreased effectiveness with repeated use as well as the possibilty of serum sickness. The development of sensitization has been linked with an increased risk of acute vascular rejection. While this association has not been reported by other centers using OKT3 prophylaxis, it is believed that the development of immune-complex disease, inadequate immunosuppression due to decreased OKT3 levels or that OKT3 sensitization may be a marker for patients at higher risk for humoral rejection may be responsible for this phenomenon.

Interleukin-2 Receptor Inhibition

A new class of drugs has been developed which targets the high affinity IL-2 receptor. This receptor is present on nearly all activated T cells but not on resting T cells. In vivo activation of the high-affinity IL-2 receptor by IL-2 promoted the clonal expansion of the activated T cell population. A variety of rodent monoclonal antibodies directed against the a chain of the receptor have been used in animals and humans to achieve selective immunosuppression by targeting only T-cell clones responding to the allograft. Chimerisation or humanisation of these monoclonal antibodies resulted in antibodies with a predominantly human framework that retained the antigen specificity of the original rodent monoclonal antibodies. A fully humanized anti-IL2R monoclonal antibody, daclizumab, and a chimeric anti-IL-2R monoclonal antibody, basiliximab, have undergone successful phase III trials demonstrating their efficacy in the immunoprophylaxis of patients undergoing renal and cardiac transplantation.

Both agents have immunomodulatory effects that are similar to those of other monoclonal antibody-based therapies (i.e., induction of clonal anergy rather than clonal deletion). The advantages of these agents include their lack of immunogenicity, long half-lives, ability to repeat dosing, and short-term safety profile. Daclizumab appears to be an effective adjuvant immunomodulating agent in cardiac allograft recipients. It has advantages over conventional induction therapy as it is more selective and can be used for prolonged and potentially repeated periods. Studies with larger cohorts are needed to further study the short-term and long-term survival benefits for patients following organ transplantation and should determine the optimal dosing schedules of these new agents.

4. STEM CELL TRANSPLANTATION AND IMMUNO-SUPPRESSION

Materno-Fetal Tolerance

As outlined above, when tissues from an HLA-disparate donor are transplanted into a recipient they are always recognized as foreign, and immunosuppression is required to prevent rejection. An important exception to this is observed in pregnant women who tolerate their unborn fetus despite the fact that it expresses a full set of non-maternal HLA antigens inherited from the father. The mechanisms by which embryonic tissue demonstrates immune privilege during prenatal development have not yet been fully elucidated, however it is evident that interactions between fetus and mother differ substantially from the events triggered by a classical allograft. Consequently, much work is being dedicated to the emerging field of materno-fetal immunobiology in order to enable the development of innovative strategies to induce tolerance and prevent allogeneic graft rejection.

When maternal T cells encounter the fetus they demonstrate adaptive tolerance. In part this may be due to the absence of expression of MHC class II antigens and low levels of expression of MHC class I antigens on fetal cells. However, this can only partly explain the state of prolonged maternal tolerance since induction of HLA class I and II molecules inevitably occurs as the fetus matures and differentiates, yet rejection still does not occur. Consequently, non-fetal aspects of the placental barrier must be of critical importance in maintaining prolonged tolerance to the fetus. An important mechanism may relate to upregulation of the human non-classical HLA class Ib antigen, designated HLA-G, by the syncytiotrophoblast. HLA-G molecules bind to inhibitory receptors on natural killer cells and subsequently protect against maternal rejection responses. The placenta produces high levels of the anti-inflammatory cytokine interleukin 10 which stimulates HLA-G synthesis while concomitantly downregulating MHC class I antigen production, thus contributing to the tolerance-inducing local environment. The trophoblast also produces high levels of the enzyme indoleamine 2,3-dioxygenase, which catabolizes tryptophan, an essential amino acid necessary for rapid T cell proliferation. Annexin II, found in isolated placental membranes in vitro is present in placental serum, exerts immunosuppressive properties, and additionally contributes to fetal allograft survival. Together, these features indicate that materno-fetal tolerance results from a combination of transiently reduced antigenicity of the fetus in combination with a complex tolerance-inducing milieu at the placental barrier.

Immunogenic Characteristics Of Embryonic And Adult Stem Cells

Murine and human embryonic stem (ES) cells do not express HLA class I and II antigens, and demonstrate reduced surface expression of co-stimulatory molecules important for T cell activation. Transplantation of murine ES cells demonstrates long-term graft survival despite the fact that these cells do acquire HLA class II antigen expression after in vivo differentiation. Since they are able to accomplish long-term engraftment without the need for immunosuppression, their inability to induce an immune response is not likely to be the result of escaping immune surveillance, but rather due to their ability to colonize the recipient thymus and induce intrathymic deletion of alloreactive recipient T cells.

Recently, a population of cells has been described in human adult bone marrow that has similar functional characteristics to embryonic stem cells in that they have high self-regenerating capability and capacity for differentiation into multiple cell types, including muscle, cartilage, fat, bone, and heart tissue. While such cells, termed adult mesenchymal stem cells (MSC), appear to have a more restricted self-renewal capacity and differentiation potential than ES cells, their functional characteristics may be sufficient for clinically meaningful tissue regeneration. A striking recent observation is that MSC can broadly inhibit T-cell proliferation and activation by various types of antigenic stimulation, including

allogeneic stimuli. MSCs have been shown to inhibit both naive and memory T cell responses in a dose-dependent fashion and affect cell proliferation, cytotoxicity, and the number of interferon gamma (IFN-gamma)-producing T cells. MSCs appear to inhibit T cell activation through direct contact, and do not require other regulatory cellular populations. Similarly to ES cells, adult bone marrow-derived mesenchymal stem cells (MSCs) do not express HLA class II molecules, and only low levels of HLA class I molecules. Despite the fact that MSC can be induced to express surface HLA class II molecules by in vitro culture with cytokines such as interferon-gamma, their ability to inhibit T cell activation results in induction of T cell non-responsiveness to the MSC themselves, endowing them with potential survival advantages in the setting of transplantation.

Tolerogenic Effects Of Stem Cell Transplantation

Extending the approaches discussed above using donor-derived blood transfusions to induce a tolerogenic state to the subsequent organ, the most promising clinical strategy for tolerance induction at present is the use of donor-derived hematopoietic stem cells in conjunction with reduced myeloablative conditioning. The objective of this therapy is to achieve a state of so-called mixed chimerism, or the permanent co-existence of donor- and recipient-derived blood cells comprising all the different hematopoietic lineages in the same host. This approach has been tested in a variety of small and large animal settings and currently available data suggest that stable engraftment of donor bone marrow reliably renders the host tolerant to donor antigens and subsequently to any cellular or solid organ graft of the same donor.

The two underlying mechanisms by which creation of a mixed-chimeric host results in tolerance induction are (1) thymic deletion of potentially donor-specific alloreactive T cells, and (2) nonthymic peripheral mechanisms, such as blocking costimulatory T cell activation, which facilitate the process of donor bone-marrow or stem cell engraftment. However, despite the efficacy of an approach using fully HLA-mismatched stem cells in an allogeneic host to induce tolerance to a subsequent organ allograft, the host is placed at a high risk of substantial morbidity and mortality due to toxicity of the myeloablative conditioning regimen and potential for graft-versus-host disease, or immune-mediated attack of the host by the implanted allogeneic stem cells.

In an attempt to overcome these potential limiting toxicities, investigators have suggested the use of either adult bone marrow-derived mesenchymal stem cells or preimplantation-derived embryonic stem (ES) cells for induction of mixed chimerism. The theoretical advantages of these cell types is their low level of surface expression of HLA class I and II antigens, and reduced surface expression of co-stimulatory molecules important for T cell activation. Rat preimplantation stage derived embryonic-like stem cells have been shown to successfully engraft in the recipient bone marrow without the need for pre-conditioning therapies such as irradiation, cytotoxic drug regimens or T cell depletion. Long-term partial mixed chimerism by use of rat preimplantation stage derived embryonic-like stem cells did not trigger graft-versus-host reactions, in contrast to the high frequency of this complication in the clinical setting of allogeneic hematopoietic stem cell transplantation. Of most interest, the induced partial chimerism enabled the recipient animals to be tolerant to a subsequent heart allograft. Allograft acceptance required the presence of an intact thymus, and rat ES cells were present in the recipient thymus.

Similar results have been reported following transplantation of human adult bone marrow-derived mesenchymal stem cells (MSC) into fetal sheep early in gestation, before and after the expected development of immunologic competence. In this xenogeneic system, human MSC engrafted, differentiated in a site-specific manner, and persisted in multiple tissues for as long as 13 months after transplantation, including the thymus. Since MSCs do not present alloantigen and do not require MHC expression to exert their inhibitory effect on alloimmune reactivity, the possibility exists that they could

theoretically be derived from a donor irrespective of their HLA type and used to inhibit T-cell responses to transplantation antigens of an unrelated third party. In initial human clinical studies, the use human adult bone marrow-derived mesenchymal stem cells has been shown to successfully enable engraftment of subsequently infused allogeneic bone marrow in transplant recipients, reduce the risk of graft-versus-host disease, and reduce the need for concomitantly administered immunosuppression. Whether similar results will be obtained when combining adult bone marrow-derived mesenchymal stem cells with solid organ allografts remains to be determined, and this is an area of active research for clinical transplant immunobiologists. Of broader relevance, if the results relating to long-term engraftment and survival of adult bone marrow-derived MSC are confirmed and extended in human clinical studies, they will have broad implications for the field of tissue and organ regeneration.

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HLA EXPRESSION IN HEMOPOIETIC DEVELOPMENT

Class I and II Antigens Are Induced in the Definitive Erythroid Lineage and Differentially Modulated by Fetal Liver Cytokines[1]

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We investigated the expression of HLA Ag on hemopoietic progenitors (burst-forming unit E, CFU-E, and CFU-granulocyte-macrophage) and precursors from human embryonic fetal liver (FL) and peripheral blood at 5 to 9 wk postconception. The expression on progenitors was evaluated by complement-mediated cytotoxicity followed by assay of residual progenitors in clonogenic culture; immunofluorescence and RIA were used for differentiated precursors.

HLA Class I and II Ag are not expressed on the primitive erythroid lineage, i.e., on yolk sac-derived megaloblasts circulating in 5- to 6-wk peripheral blood. However, they are gradually induced on the definitive erythroid lineage in FL. Their expression on progenitors is first detected at 6 wk and shows a progressive increase through 9 wk, up to ≥50% of adult values. A similar expression pattern is observed for FL erythroblasts. Incubation of 6-wk FL erythroid cells with IFN-α, -β, or -γ, or TNF-α induces a sharp rise of the expression of HLA class I, but not HLA class II Ag on both progenitors and precursors. In contrast, incubation with PHA-stimulated adult leukocyte-conditioned medium causes a marked increase of both HLA-ABC and HLA class II Ag expression. We hence investigated the effect of cytokines present in leukocyte-conditioned medium on the expression of HLA class II Ag: although IL-1α, IL-2, and granulocyte-macrophage-CSF do not exert a significant action, IL-1β and IL-4 induce a marked increase of HLA class II, but not class I, Ag expression on 6-wk FL progenitors and precursors. Low amounts of IFN-γ, TNF-α, and IL-1β were detected in the supernatants and extracts of 6-wk FL cells. The concentrations of these cytokines in both supernatants and extracts sharply increase in the 7- to 8-wk period, particularly for TNF-α and IL-1β, thus indicating a direct correlation with the rise of HLA Ag expression on FL erythroid cells in the same period. In conclusion, the expression of HLA class I and II Ag is not detected on primitive megaloblasts, but is gradually induced on definitive FL progenitors and precursors, possibly via production of specific cytokines in the FL microenvironment, i.e., IFN-γ and TNF-α for class I Ag and IL-1β for class II Ag.

The human MHC codes for HLA class I and II Ag. HLA class I Ag (HLA-A, -B, and -C) are expressed on the membranes of the majority of nucleated cells, whereas HLA class II Ag (HLA-DR, -DQ, and -DP) are present on both immune system cells (i.e., macrophages and other APC, B lymphocytes, and activated T lymphocytes) and other cell types, including endothelial cells, fibroblasts, and epithelial cells (1). HLA class II Ag play a key role in cellular interactions mediating Ag recognition and the immune response (2, 3).

The level of HLA expression on a variety of cells in culture is positively modulated by addition of IFN (4–6). Particularly, IFN-γ enhances or induces the expression of HLA class I Ag on fibroblasts (5) and class II Ag on monocytes (7, 8), endothelial cells (9, 10), fibroblasts (11), astrocytes (12), and keratinocytes (13). Similarly, TNF-α and IL-4 positively modulate the expression, respectively, of HLA class I or II Ag on B cells and macrophages (14, 15) or fibroblasts (16).

In human adult hemopoiesis, HLA class I Ag are expressed on both undifferentiated progenitors and morphologically recognizable precursors (17–25). HLA class II molecules are highly expressed on early progenitors, i.e., pluripotent (CFU-granulo-erythro-mono-megakaryocytic), BFU[3]-E), and day 14 CFU-GM progenitors (17–25). Their expression, however, is sharply diminished and undetectable, respectively, in late progenitors and differentiated precursors of both the erythroid (CFU-E, erythroblasts) and granulocytic pathway (day 7 CFU-GM, GM precursors) (17, 18); on the basis of these findings the possibility exists that HLA class II Ag may play a specific role in hemopoietic differentiation, which is not related to the immune response (17, 18).

Less information is currently available on MHC Ag expression during ontogenic development, particularly in the hemopoietic system. In the murine species, class I Ag

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[2] Correspondence and reprint requests should be sent to: M. Gabbianelli, Department of Hematology-Oncology, Istituto Superiore di Sanità, Viale Regina Elena, 299-00161 Rome, Italy.
[3] Abbreviations used in this paper: BFU, burst-forming unit; GM, granulocyte-macrophage; YS, yolk sac; FL, fetal liver; IMDM, Iscove's modified Dulbecco's medium; LCM, leukocyte-conditioned medium; Ep, erythropoietin.

are not detected during the early stages of embryonic development, but their expression is progressively acquired during fetal life (26). In human mid- and late-gestation HLA class I and II Ag expression shows a gradual rise up to adult values on a variety of nonhemopoietic cells (27). HLA class II Ag are not present on placenta macrophages in the first trimester, but become gradually expressed from the second trimester onward (28). Not surprisingly, the mechanisms controlling HLA expression during development are still obscure. IFN-γ induces class I Ag on murine embryonic and human amnion cells (26, 29), but not class II molecules on teratocarcinoma cells (30).

We have analyzed the expression of HLA class I and II Ag on primitive megaloblasts derived from the YS, compared with definitive erythroid progenitors and precursors in 5- to 9-wk embryonic FL. Thereafter, we investigated the in vitro modulation of HLA Ag on 6-wk FL erythroid cells induced by recombinant cytokines, compared with the production of these cytokines by FL cells. The results indicate that HLA class I and II Ag, although absent on megaloblasts, are gradually induced in the definitive lineage and are differentially modulated by specific FL cytokines.

MATERIALS AND METHODS

Cells. Human embryos and fetuses at 5- to 9-wk postconception were obtained from legal curettage abortions after fully informed consent. Gestational age was accurately evaluated as described elsewhere (31). Cord or heart blood was obtained by aspiration from 5- to 6-wk embryos. FL was sterilely dissected under a stereoscopic microscope. Monocellular suspensions of FL cells in IMDM were depleted of hepatocytes and macrophages by spontaneous sedimentation for 30 min at room temperature, followed by 1-h incubation in petri dishes to remove adherent elements (further details in Ref. 31).

RNA extraction and analysis. Total cellular RNA was extracted from fresh or liquid nitrogen frozen FL by the guanidium isothiocyanate technique (32) and analyzed by dot or Northern blotting (33). Total RNA (8.0- to 0.5-μg aliquots) was dotted onto nitrocellulose paper and hybridized with HLA class I and HLA-DR cDNA probes (34, 35). For Northern analysis, 5 μg of total RNA were run on 1.0% formaldehyde agarose gel, transferred onto nylon membranes by capillary blot, and hybridized as described for dot blots. Hybridization to a chicken β-actin probe (36) was carried out to normalize RNA levels.

Human recombinant or highly purified cytokines and LCM. Highly purified IFN-β was kindly provided by Biogen (Geneva, Switzerland) and rIFN-α by Euro-Roche (Milan, Italy). rIFN-γ and TNF-α were obtained from Boehringer Ingelheim (Wien, Austria). rGM-CSF, IL-2, IL-1α and IL-1β, and IL-4 were purchased from the Genetics Institute (Cambridge, MA), Boehringer (Mannheim, W. Germany), Janssen (Beerse, Belgium) and Genzyme (Boston, MA), respectively.

LCM was obtained from human adult PBMC stimulated by PHA (Sigma, St. Louis, MO, 5 μg/ml) for 48 h as previously described (37).

mAb. The following mAb were used in the cytotoxicity assay: Tec-HLA-ABC (Technogenetics, Turin, Italy), Tec-HLA-Ia (Technogenetics) and OKIa (Ortho Diagnostic System, Milan, Italy), which recognize monomorphic epitopes of human HLA-ABC, and HLA class II Ag, respectively.

In the immunofluorescence and immunoradiometric assays, the following mAb were utilized: Tec-HLA-ABC, Tec-HLA-DR, Tec-HLA-DQ, Tec-β-microglobulin (Technogenetics), and anti-HLA-DR, -DQ, and -DP (Becton Dickinson, Milan, Italy), which recognize a monomorphic epitope of human HLA-DR, -DQ, -DP and β_2-microglobulin Ag.

Indirect immunofluorescence and immunoradiometric assays. HLA Ag expression on FL precursors was evaluated by either indirect immunofluorescence (percentage of positive cells) or immunoradiometric assays. In both procedures, cells were incubated first with an appropriate concentration of mAb and then with either fluorescent isothiocyanate-labeled F(ab')$_2$ fragments of immunoadsorbent-purified sheep antibodies against mouse IgG (immunofluorescence) or ^{125}I-F(ab')$_2$ anti-mouse IgG (immunoradiometric assay).

Cytotoxicity test and assay for hemopoietic progenitors. The reactivity of progenitors with anti-HLA mAb was assessed by a complement cytotoxicity assay as described elsewhere (23, 38).

Briefly, FL cells were incubated with an optimal concentration of mAb at 4°C for 40 min. Rabbit C (Behring Institute Scoppito, L'Aquila, Italy) was added and the incubation continued at 37°C for 90 min. Control cells were incubated with: (a) medium alone, (b) medium, and C, (c) medium and mAb. Preliminary experiments with graded concentrations of C and mAb were performed to establish the optimal concentrations of both reagents. For these experiments a single batch of rabbit C was selected. Careful controls showed that this batch by itself was not cytotoxic for FL cells. Furthermore, control experiments showed that at the end of the cytotoxicity assay, FL cells incubated with anti-HLA mAb and C were completely depleted of HLA$^+$ cells, whereas HLA$^-$ cells were unaffected by this procedure. After the cytotoxicity test, FL cells were plated in methylcellulose culture: each dish contained 10^5 cells in 1 ml of IMDM (Gibco, Scotland) supplemented with 0.8% methylcellulose and either (1) 40% FCS (Flow, Irvine, Scotland) and 1.5 IU human recombinant Ep, (Amgen, Thousand Oaks, CA), or (2) 30% FCS and 10% giant cell tumor-conditioned medium (GIBCO) as a source of GM-CSF, in absence of Ep. Each experimental point was performed in duplicate. The dishes were incubated at 37°C in a fully humidified 5% CO_2 air atmosphere. CFU-E-derived clusters were scored on day 4, BFU-E-generated bursts and CFU-GM-derived colonies on days 8 to 10 (31).

The reactivity of antibodies with fetal human hemopoietic progenitors was evaluated in terms of the number of colonies generated by samples incubated with both anti-HLA antibodies and nontoxic rabbit C, expressed as percentage of the mean values of controls treated with either antibody or C alone.

Incubation of FL cells with LCM and recombinant cytokines. FL cells (6-wk) were incubated with 10% LCM or 50 to 100 IU/ml of IFN-α, -β, or -γ in IMDM containing 10% FCS for 20 h (preliminary experiments showed similar results after a 20- or 40-h incubation period). The other cytokines (IL-1 α or -1β, IL-4, TNF-α, and GM-CSF) were similarly tested at graded concentrations, as specified below. Control samples were incubated with IMDM/FCS alone. The reactivity of hemopoietic progenitors and precursors with anti-HLA mAb was then evaluated as described.

In one experiment, the IFN-γ contained in LCM (100 U/ml) was neutralized by addition of an excess (500 anti-U/ml) of a neutralizing anti-IFN-γ mAb (Eurogenetics, Turin, Italy).

Ep, IL-1β, TNF-α, and IFN-γ concentrations in supernatants and lysates of FL cells. FL cells from 6-, 7-, or 8-wk embryos were incubated for 24 h in IMDM supplemented with 10% FCS. Thereafter, the culture supernatant was recovered, centrifuged at 13,000 rpm for 10 min and stored at −80°C.

Lysates of 6-, 7-, or 8-wk FL cells were obtained by freeze thawing in hypotonic buffer (Tris 10^{-3} M, pH 7.20, 3.5 U/ml of trypsin inhibitor aprotinin). Lysates were centrifugated at 25,000 rpm for 15 min. The supernatant was recovered and stored at −80°C.

Ep concentration was evaluated by ELISA with recombinant human Ep as the standard (Clinical Research Corporation, Knoxville, TN). IFN-γ concentration was estimated by RIA (Centocor, Malvern, PA), which allows the detection of 0.05 U IFN-γ/ml. The level of IL-1 was assessed by a sensitive ELISA (Cistron Biotechnology, Pine Brook, NY), which can detect as little as 10 pg/ml. Finally, the concentration of TNF-α was evaluated by ELISA, which detects levels as low as 10 pg/ml (T Cell Sciences, Cambridge, MA). Based on these assays, Ep, IFN-γ, IL-1β, and TNF-α were not detected in IMDM supplemented with 10% FCS.

RESULTS

To determine the time course of HLA expression on hemopoietic cells during ontogenic development, we analyzed the expression of surface class I and II Ag on: 1) megaloblasts from 5- to 6-wk cord or heart blood, which pertain to the primitive lineage derived from the YS and 2) hemopoietic progenitors and precursors from 5- to 9-wk FL, which pertain to the definitive macrocytic lineage.

HLA expression on YS-derived megaloblasts vs FL erythroblasts. In a first set of experiments, we evaluated the HLA expression on primitive and definitive erythroid precursors by immunofluorescence techniques. Primitive megaloblasts from 5- to 6-wk circulating blood (> 99% pure) (39) do not react with anti-HLA-ABC and -DR mAb (Table I). As a positive control, FL precursors from 6- to

8-wk human embryos showed a progressively increasing sensitivity to anti-HLA-ABC and -DR (Table I).

HLA expression on FL hemopoietic progenitors and precursors. HLA expression on hemopoietic progenitors was evaluated after incubation of FL cells with two mAb recognizing monomorphic epitopes of HLA-ABC and HLA class II antigens in a standard cytotoxicity complement-mediated assay, followed by cloning of erythroid (BFU-E, CFU-E) and CFU-GM progenitors in methylcellulose culture (Fig. 1, *A* and *B*).

At 5 wk, BFU-E and CFU-GM apparently do not express class I and II Ag (CFU-E were not evaluated, because in 5-wk FL they are present only in very low numbers) (3). At 6 wk, however, a minority (20 to 25%) of BFU-E, CFU-E, and CFU-GM express these Ag. The percentage of reactive progenitors progressively increased up to 9 wk: a more rapid increase was observed for CFU-GM as compared to BFU-E and CFU-E. However, even at 9 wk, the positivity of hemopoietic progenitors with class I and II HLA mAb is significantly lower than corresponding adult levels, i.e., > 90 to 95% reactivity (17, 18). It is emphasized that a similar level of class II Ag expression was observed on both early (BFU-E) and late (CFU-E) FL erythroid progenitors: in adult hemopoiesis, a sharp decline of the expression is observed during differentiation of BFU-E to CFU-E (17, 18).

TABLE I

Reactivity of primitive YS-derived megaloblasts vs FL definitive erythroblasts with anti HLA-mAb[a]

mAb	Primitive YS Megaloblasts (% Positive Cells)	Definitive FL Erythroblasts (% Positive Cells)		
		6 wk	7 wk	8 wk
Anti-HLA-ABC	<0.1	5	9	17
Anti-HLA-DR	<0.1	2	5	13

[a] Data represent the mean value from three separate experiments.

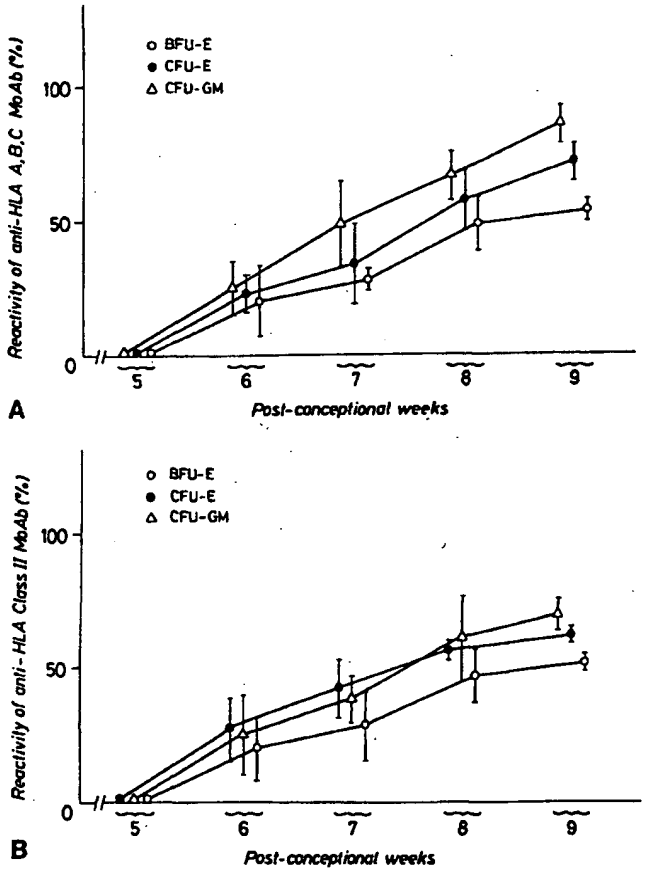


Figure 1. (*A* and *B*) Reactivity of embryonic FL hemopoietic progenitors (BFU-E, CFU-E, and CFU-GM) of different ontogenic ages with anti-HLA-ABC and class II mAb. FL were obtained from 5- to 9-wk human embryos. The reactivity of anti-HLA mAb with hemopoietic progenitors was evaluated by a complement-dependent cytotoxicity assay. The number of hemopoietic progenitors was evaluated by using a standard clonogenic assay in methylcellulose. The values represent the mean level from five separate experiments.

The presence of HLA Ag on the membrane of more differentiated hemopoietic precursors was evaluated using FL erythroid cells at different ontogenic stages by immunoradiometric assay with anti-HLA mAb and ^{125}I-F(ab')$_2$ anti-mouse Igs antibody. This analysis showed that HLA-ABC and -DR Ag expression is very low in 6-wk FL precursors, then rises at 7 wk up to peak levels at 8 wk (Fig. 2).

RNA expression in FL cells. Total RNA from whole FL was analyzed by dot blot with HLA class I and DR cDNA probes. The time-course of RNA expression is shown in Figure 3. A low level of class I transcripts is present in 5-wk FL, i.e., 1 wk before the appearance of the corresponding Ag on the erythroid cell surface. This parameter shows a sharp increase at 6 wk and a slow decline in subsequent weeks. In contrast, FL class II RNA expression exhibits a gradual rise from 6 through 8 wk.

Modulation of HLA expression on hemopoietic progenitors and precursors. We initially investigated HLA expression on 6-wk FL hemopoietic progenitors after incubation with IFN-α, -β, -γ, TNF-α, and LCM (Fig. 4 and results not shown). All types of IFN and TNF-α induce a marked increase in the expression of class I, but not class II, Ag, i.e., the reactivity of anti-HLA-ABC antibody rises

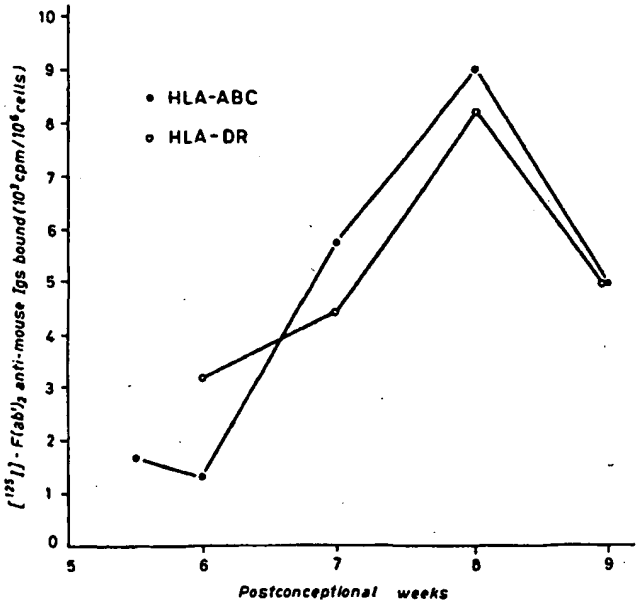


Figure 2. Expression of class I and II HLA Ag on FL hemopoietic precursors derived from 5- to 9-wk embryos. HLA expression was evaluated on total FL cells by an immunoradiometric assay using specific mAb which recognize nonpolymorphic epitopes of HLA-ABC and HLA-DR Ag, respectively. The values represent the mean level from three separate experiments.

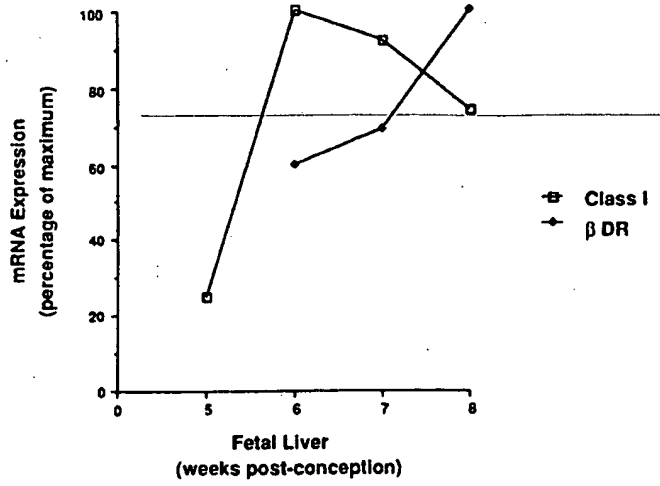


Figure 3. Expression of RNA encoding HLA-ABC and -DR in FL cells from 5- to 8-wk human embryos. Total RNA was extracted by the guanidinium thiocyanate technique and decreasing amounts of total RNA (10 to 0.5 μg) were dotted onto nitrocellulose paper and then hybridized to 10^7 cpm of nick-translated HLA class I and II cDNA probes, respectively. Appropriately exposed autoradiograms were analyzed by densitometric scanning. The results, after normalization with β-actin, are represented as the percentage of maximum expression level.

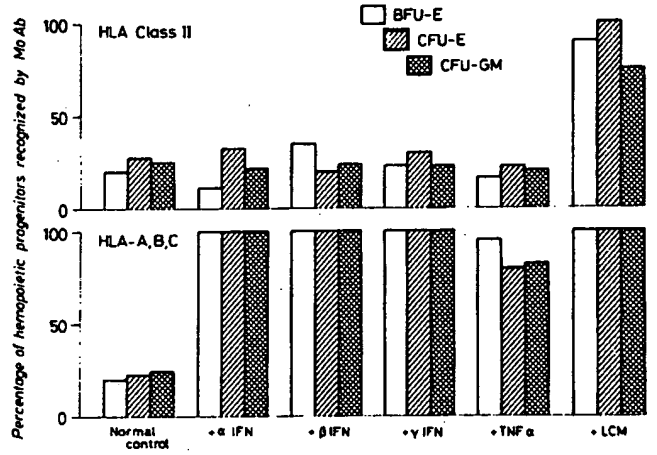
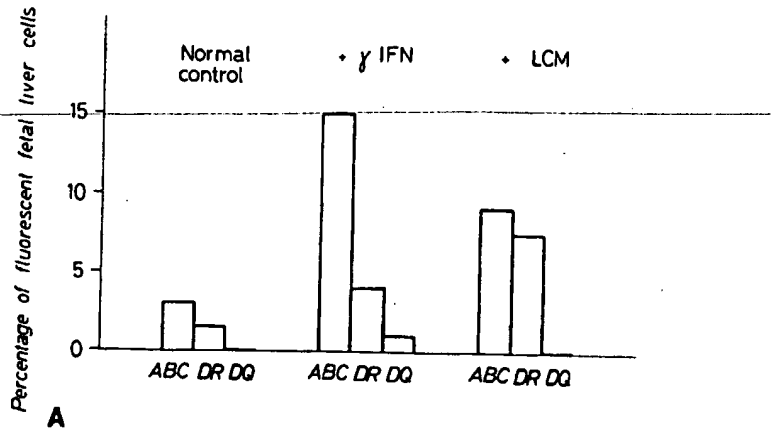


Figure 4. Effects of several cytokines IFN-α (100 U/ml), -β (100 U/ml), -γ (100 U/ml), TNF-α (10 U/ml) and PHA-stimulated human adult LCM (10%) on the expression of HLA class I and II Ag on 6-wk FL hemopoietic progenitors. FL cells were incubated for 20 h in the presence of each cytokine or LCM. Expression of HLA Ag on progenitors was then evaluated by means a complement-dependent cytotoxicity assay with anti-HLA specific mAb. Mean values from three separate experiments are presented.

from 20 to 25 up to 100%, whereas that of anti-HLA class II antibody remains essentially unchanged at 25%. On the other hand, LCM causes a marked increase of the expression of both HLA class I and II Ag (from 25 to 100%).

The same experiment was performed on 6- to 7-wk FL hemopoietic precursors. FL cells depleted of hepatocytes and macrophages were grown for 24 h in absence or presence of either IFN-γ or LCM. HLA expression was then evaluated by indirect immunofluorescence (Fig. 5*A*) and immunoradiometric assay (Fig. 5*B*). The results are in line with those observed for progenitor cells, i.e., IFN-γ selectively stimulates the expression of HLA-ABC Ag, whereas LCM enhances both HLA-ABC and -DR. We also observed that HLA-DQ is expressed in a very low percentage of FL hemopoietic precursors: this Ag is not



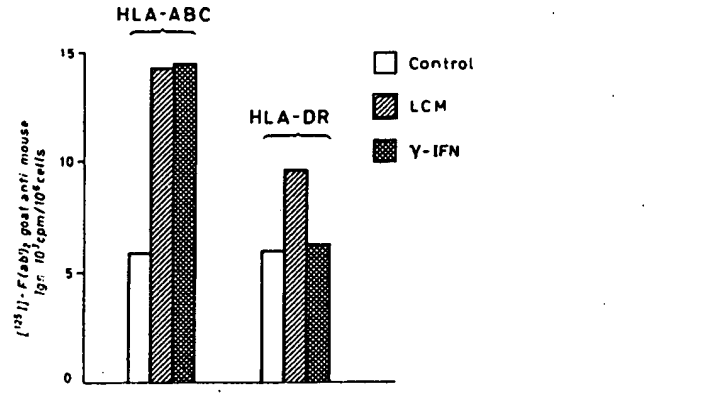


Figure 5. (*A* and *B*) Modulation of HLA-ABC and -DR Ag expression on embryonic-FL erythroid precursors by IFN-γ (100 U/ml) and LCM (10%). FL cells were obtained from a 7-wk embryo and HLA expression was evaluated by immunofluorescence (*A*) or immunoradiometric assay (*B*). Mean values from three separate experiments are presented.

modulated by treatment with IFN-γ and LCM (Fig. 5*A*).

The possibility was considered that the enhancing effect of LCM on the expression of class I Ag may be ascribed to IFN (particularly IFN-γ) and/or other cytokines present in LCM.

We first assayed the concentration of IFN-γ in LCM by a sensitive RIA. Thereafter, a fivefold excess of a neutralizing anti-IFN-γ mAb was added to the medium: this caused a complete inhibition of the enhancing effect of LCM on HLA-ABC expression, whereas the stimulatory effect on HLA class II Ag expression was unmodified (results not shown).

In an attempt to identify the factor(s) in LCM responsible for selective HLA class II modulation, we investigated the effect of several cytokines presumably present in LCM on the expression of class II Ag (Fig. 6 and results not shown). Although GM-CSF, IL-2, and IL-1α did not exert a significant action at several concentrations (not shown), both IL-1β (10 U/ml) and IL-4 (20 U/ml) induced a marked increase of HLA class II Ag on 6-wk FL progenitors (Fig. 6).

Assay of cytokines modulating HLA expression in supernatants and lysates of FL cells (Table II). The presence of cytokines modulating HLA Ag was evaluated in supernatants and lysates of 6-, 7-, and 8-wk FL cells. TNF-α and IL-1β are present at low levels in the supernatants and lysates of 6-wk FL. Their concentration

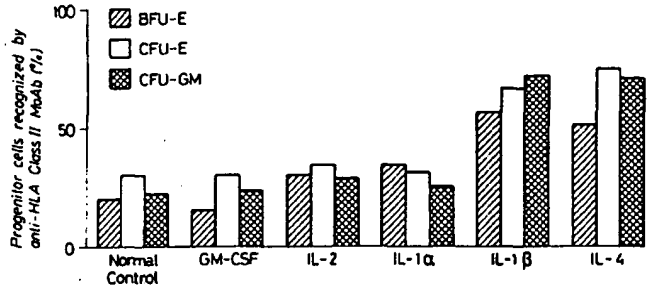


Figure 6. Modulation of HLA class II Ag expression on FL progenitors by recombinant cytokines GM-CSF (10 ng/ml), IL-2 (100 U/ml), IL-1α and -1β (10 U/ml), IL-4 (20 U/ml). FL progenitors were derived from 6-wk-old embryos. The reactivity of hemopoietic progenitors (BFU-E, CFU-E, and CFU-GM) with anti-HLA class II Ag was evaluated by a complement-dependent cytotoxicity assay. Mean values from three separate experiments are presented.

shows an increase at 7 wk and a further rise at 8 wk (Table II). Thus, the rise of TNF-α and IL-1β concentrations is directly related to the increase of class I and II Ag expression on progenitors and precursors in the same developmental period. The concentration of IFN-γ is low in 6-wk FL lysates and supernatants, but shows a two-fold rise at 8 wk (Table II).

It is known that the FL is the main site of Ep production in fetal life (40,41): as a control, the concentration of Ep was therefore evaluated in FL samples. In the 6- to 8-wk embryonic period the concentration of this factor is essentially unmodified in supernatants, although showing almost a two-fold increase in lysates (Table II).

DISCUSSION

MHC class I and II Ag represent a complex series of cell surface determinants, which play a key role in immunoregulation and cell-cell interaction in the immune response. In addition, HLA class I and II Ag may function in a broader, nonimmune context, as indicated by the modulatory effect of anti-HLA class I mAb on the proliferation of T cells stimulated with various mitogens. Furthermore, class II Ag have been detected on endothelial cells (9, 10), fibroblasts (11) and epithelial cells (3): it has been suggested that they may be involved in other types of cell-cell interaction(s), such as cell adhesion (3, 42, 43).

As previously mentioned, maturation of adult hemopoietic progenitors to morphologically recognizable precursors in the erythroid and GM pathway is associated with a gradual loss of class II HLA expression (17, 18). Differential expression of HLA-DR, -DQ and -DP on various hemopoietic progenitors has also been demonstrated (24, 25, 44–46). More specifically, class II Ag have been implicated in the in vitro regulatory interactions between T cells and BFU-E (46). The suppressor action of T cells on BFU-E is genetically restricted at HLA class II level, in both normal conditions (47) and erythroid aplasia (48). On the other hand, HLA class II expression on CFU-GM correlates with the in vitro inhibitory effect exerted by acid isoferritins or PGE on the clonogenic capacity of these progenitors (49).

HLA expression on human hemopoietic cells, although extensively analyzed in adult life, has not been investigated during the embryonic-fetal period. In this regard, ontogenic development of hemopoiesis involves a series of coordinated changes in embryonic and early fetal life (i.e., at 3 to 8 wk and 9 to 10 wk postconception, respectively), as detailed by systematic analysis of embryonic-fetal hemopoietic cell kinetics at precise sequential times (31, 39, 50). At 3 to 4 wk the first generation of hemopoietic cells proliferates in the YS and extraembryonic mesenchyme. These elements consist largely of primitive erythroblasts (megaloblasts), which are present in circulating blood from 4 wk onward. At 5 to 6 wk, the YS is replaced by the FL as the main hemopoietic site. In FL parenchyma, definitive erythropoietic cells massively proliferate, thus giving rise to enucleated macrocytes. The definitive series enters into the bloodstream (31, 51) from 8 wk onward, thus gradually replacing circulating megaloblasts. It is still debatable whether a single clone of stem cells migrates from the YS to colonize the FL via the bloodstream (31, 52) and/or two independent clones of hemopoietic stem cells are generated by undifferentiated cells in YS and then in FL (53).

This study represents the first analysis of the expression of HLA Ag on human hemopoietic progenitors and precursors during embryonic and early fetal life. The results suggest that in these cells expression of both HLA-ABC and HLA class II molecules is under developmental control, at least in part via release of specific cytokines in the FL microenvironment.

Immunofluorescence analysis showed that primitive YS-derived megaloblasts in 5- to 6-wk circulating blood are HLA-negative. Accordingly, HLA is not expressed in 5-wk FL erythroid precursors (i.e., before the initiation of definitive FL erythropoiesis), which are essentially represented by primitive megaloblasts in liver sinusoids (36, 54, 55).

The expression of HLA class I and II Ag is switched on precisely at the time of initiation of definitive FL erythropoiesis. Thus, HLA class I and II molecules are not detected on 5-wk FL progenitors. However, they are expressed at low levels in 6-wk FL progenitors and precursors: at this stage definitive CFU-E and macrocytic erythroblasts start to differentiate from BFU-E in FL (36, 53). The expression of HLA class I and II Ag on FL progenitors then shows a gradual rise from 6 through 9 wk, up to ≥50% of adult values. A similar pattern is observed for FL erythroid precursors. In line with our findings, previous observations on later developmental stages indicate

TABLE II

Ep, IFN-γ, TNF-α, and IL-1β concentrations in supernatants and lysates of FL cells at different postconception ages[a]

Time Postconception (wk)	Ep		IFN-γ	TNF-α		IL-1β	
	Supernatant (mU/ml)	Lysate (mU/10^6 Cells)	Supernatant (U/ml)	Supernatant (pg/ml)	Lysate (pg/10^6 Cells)	Supernatant (pg/ml)	Lysate (ng/10^6 Cells)
6	32	35	0.105	12	ND	72.5	1.4
7	38	40	NA	35	11	127	2.3
8	40	60	0.225	100.5	24	247	3.6

[a] Data represent mean values from three separate experiments. NA, not available.

that HLA Ag are gradually expressed on a variety of fetal nonhemopoietic tissues, up to adult values at 24 to 32 wk postconception (27).

The induction of HLA expression in the definitive lineage is of uncertain functional significance. Hypothetically, it may be related to the onset of mechanism(s) controlling the differentiation of the definitive erythroid lineage in the FL microenvironment. In this regard, the differentiation program of stem cells undergoes a series of coordinated switches during development from the YS "primitive" stage to the FL "definitive" one, as evaluated at the level of primitive vs definitive erythroblasts. The switches pertain to both cell morphology (megaloblasts → macrocytes) and differentiation markers (e.g., embryonic → fetal Hb, $HLA^- \rightarrow HLA^+$ hemopoietic cells). It is of interest that in FL erythropoiesis, HLA class II Ag are expressed not only on BFU-E, but also on CFU-E and erythroblasts, thus in sharp contrast with their selective expression on early progenitors in adult erythropoiesis (21, 22).

Our in vitro results indicate that class I and II Ag on FL hemopoietic progenitors and precursors are positively modulated by different cytokines. Thus, IFN-γ and TNF-α enhance the expression of class I Ag, whereas IL-1 beta and IL-4 enhance that of class II molecules. As previously mentioned, IFN (especially IFN-γ) are strong inducers of MHC class I and II Ag on a variety of human adult cell types (5, 7–13), as well as on class I Ag on murine embryonic and human amnion cells (26, 29). Furthermore, TNF-α and IL-4 positively modulate, respectively, HLA class I and II Ag on fibroblasts (16) or B lymphocytes and macrophages (14, 15). We first report here the modulating effect of IL-1 beta on HLA class II expression.

More importantly, the concentration of the cytokines modulating HLA expression on embryonic hemopoietic cells has been assayed in supernatants and extracts of FL cells at 6 through 8 wk postconception. The results neatly correlate with the time course of expression of MHC Ag on definitive hemopoietic cells. Very low amounts of IFN-γ, TNF-α, and IL-1 have been assayed in 6-wk FL cells, when MHC class I and II Ag are first detected on both progenitors and precursors. At 7 to 8 wk the sharply increased concentrations of these molecules, particularly TNF-α and IL-1β, are associated with the rise of HLA Ag expression on the definitive erythroid lineage. In line with these findings, recent studies show a developmental control of the expression of lymphokines in human thymus (54) and IFN in hamster embryonic tissues (55).

In conclusion, we suggest that the gradual induction of HLA expression on definitive FL hemopoietic progenitors and erythroblasts is modulated by progressive release of specific cytokines in the hepatic microenvironment.

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